Evaluation Of Hydroalcoholic Root Extract Of *Withania somnifera* Against Propionic Acid Induced Autism Spectrum Disorder In Mice

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MASTER OF PHARMACY

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By

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UNDER THE GUIDANCE OF

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This is to certify that Project entitled **Evaluation Of Hydroalcoholic Root Extract Of *Withania somnifera* Against Propionic Acid Induced Autism Spectrum Disorder In Mice** submitted by Register No: **261425016** in partial fulfilment of the course for the award of the degree of **Master of Pharmacy in Pharmacology.** It was carried out at Department of Pharmacology in C.L. Baid Metha College of Pharmacy, Chennai-97 under my guidance during the academic year 2015-2016.

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DECLARATION

1. **Register No. 261425016,** hereby declare that this dissertation entitled, **Evaluation Of Hydro Alcholic Root Extract Of *Withania somnifera* Against Propionic Acid Induced Autism Spectrum Disorder In Mice ‘**, has been originally carried out by me under the guidance and supervision of **Prof. Dr.P.Muralidharan, M.Pharm,. PhD,** Head of the department of pharmacology, C.L. Baid Metha College of Pharmacy, Chennai-97 for the academic year 2015-2016. This work has not been submitted in any other degree at any other university.

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LIST OF ABBREVATIONS

α	Alpha
β	Beta
γ	Gamma
E	Epsilon
€	Euro
%	Percentage
µ	Micro
µl	Micro litter
&	And
5-HT	5-hydroxy tryptomine

8OHdG	8–hydroxy-2deoxyguanosine
Ach	Acetylcholine
AchE	Acetylcholine estrase
ADHD	Attention deficit hyperactivity disorder
AS	Asperger syndrome
ASD	Autism spectrum disorder
B.C	Botanical classification
BBB	Blood Brain Barrier
BDV	Borna Disease virus
CAT	Catalase
CAM	Complementary and alternative medicine
CPCSEA	Committee for the purpose of Control and Supervision of Experiments on Animals
CNS	Central Nervous System
CDD	Childhood Disintegrative Disorder

Cm	Centimeter
ºC	Degree Celsius
DNA	Deoxy-ribo Nucleic Acid
DCD	Developmental Coordination Disorder
DSM	Diagnostic and Statistical Manual
EDTA	Ethylene Diamine Tetra Acetic acid
E/I	Excitatory/inhibitory
EL	Escape latency
FDA	Food and Drug Administration
g.	Gram
GP_X	Glutathione Peroxidase
GRD	Glutathione reductase
GSH	Glutathione
HAREWS	Hydro-alcoholic root extract of *Withania somnifera*
HCARE	High-concentration Ashwagandha root extract

HCl	Hydrochloric acid
HP	Haloperidol
H_2O_2	Hydrogen peroxide
ID	Intellectual disability
IAEC	Institutional Animal Ethics Committee
IL	Interleukin
i.c.v.	Intracerebro ventricular
i.p	Intraperitoneal
Kg	Kilogram
MCT	Monocrotanile
MDA	Malonyldialdehyde
Mg	*Milligram*
ml.	*Milliliter*
mM	*Milimolar*
mGluR	metabotropic glutamate receptors

MMR	measles, mumps, rubella
MWM	Morris Water Maze
NADH	Reduced Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced Nicotinamide adenine dinucleotidephosphate
ng	*Nanogram*
nm	*Nanometer*
OECD	Organization for Economic Co-operation and Development
OH	Hydroxide
OPT	O-phthaldialdehyde
PPA	Propionic acid
PDD-NOS	Pervasive Developmental Disorder Not Otherwise Specified
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
Pg	Pico gram

PI	Phosphatidylinosito
PMSF	phenyl methyl sulpfonyl fluoride
Po	Post oral
PS	Phosphatidylserine
SDS	Sodium dodecyl sulphate
Sec	Seconds
SEM	Standard Error Mean
SOD	Superoxide dismuthse
SM	Sphingomyelin
TBA	Thiobarbituric acid
TL	Transfer Latency
TNF	Tumor necrosis factor
TNBS	Trinitro Benzyl Sulfonic Acid
WHO	World Health Organization

1. INTRODUCTION

Nature is the delightful example for the phenomena of symbiosis. Natural products are origin from plants, animals, metals and minerals serving as the basis for the treatment of human disease. Medicinal plants based on tradition system of medicine have been playing an incredible role in providing diagnosis and treatment of human beings especially in developing countries. Utilization of herbal drug has also increased in developed countries. (1)

Herbal drug is the oldest form of health care known to mankind. Herbs had been used by all the cultures throughout the history. In modern civilization herbal drug is an integral part of the development. Primitive man observed and appreciated the great diversity of plants available to him. The most use of medicinal plant has been developed through observation of wild animal by trials and errors. As time moved on, each tribe added the medicinal power of herbs in their area based on their knowledge. They collected the information on herbs based on the method and well-defined it in herbal pharmacopoeia. Indeed, well into the 20th century most of the pharmacopoeia of scientific medicine was derived from the herbal lore of native place. Much of the drug commonly use now a day is of herbal origin. Most civilized country USA dispensed about 25% of prescription which contains at least one active ingredient derived from plant materials. Some are made from plant extract others are synthesized to mimic the natural plant compounds. (2)

From last five thousand years human being has relied on natural product as the primary source of medicines. However, the last two centuries have brought an explosion to understand how the natural products are produced and how they react with other organisms. The World Health Organization (WHO) estimates that 80% of the world health populations presently use herbal medicines for some aspect of primary health care (3).

In recent years synthetic drugs are showing more adverse affect, to overcome this problem researchers are trying to avoid this risk of those drugs. Whenever a drug is prescribed to a patient they are facing risk of side effect, so long term use of these drugs patient should be careful. But in herbal medicine the toxic effects are negligible, so the uses of herbal industry are growing up. Indian, Chinese are using plant as medicine, as whole

plant or its extract. Toxicity of herbal drugs is less when compared with the synthetic medicines.[4]

India is known as a botanical garden in world and the largest producer of herbal medicines. India recognizes more than 3000 plant as medicinal use. It is estimated that more than 6000 plants in India are in use in traditional and herbal system of medicines. Herbal medicines are used in various forms in indigenous system such as Unani, Ayurveda, and Siddha.[5]

Around 25,000 effective herbal formulations are used in traditional and folk medicine in India. The demand for plant products is increased throughout the world and the pharmaceutical companies are currently carrying out research on plant material for the potential medicinal components. Even though they are not able to prove the therapeutic effects of many plants, research continues to screen the active ingredients which form the basis of drugs to fight disease like psychological disorder, neuro-developmental disorder, diabetes, cancer, AIDS and various more chronic disease[6].

In past years, the use of herbal drugs against various diseases as they are commonly non-toxic and have less side effects has developed. Even the World Health Organization (WHO) has recommended the effective use of plants in conditions wherever modern drugs are not safe [7]. Sometimes, herbal preparation produces a good therapeutic response when given in combination with allopathic drugs.

The CNS disorders cause a range of complex, distress and life threatening symptoms some of which are not at all responsive. They generally leave the patient unable to function normally certain neuro transmitters cause neuro disorders such as ASD, Parkinsonism disease, myasthenia gravis, attention deficit disorders, epilepsy, anxiety myotropic lateral disease, multiple sclerosis[8].

It is very important to use these medicines in a rational way. On basis of traditional knowledge about medicinal use of plant as therapeutic agents a rational approach has been developed to use medicinal plants as lead for discovery of active molecules e.g. Gingko biloba extract can be as CNS stimulant[9].

In the modern research literature a number of clinical and experimental studies examine the potential herbs and formulae for ASD and related conditions. The classical literature contains herbal treatments for disorders that have symptoms and signs similar to those of ASD by combining and systematically evaluating the data derived from the modern and classical literature, it is expected that the herbs and formulae with the greatest potential for further research can be identified.

Considering the advantages of herbal drugs over the modern medicine and very keen to conserve the Indian traditional use of herbal products, the current project is designed, choosing the medicinal plant against autism spectrum disorder, the neuro-developmental disorder, where no proper evidence has been registered in use of herbal medicine.

1.1. Neurodevelopment disorder[10,11]:

Impairment growth and development of brain or central nervous system are known as neurodevelopment disorders. This term refers to a disorder of brain function that affects emotion, memory, self-control and ability of learning and that unfolds the individual grows. This term is exclusively used as a synonym of Autism and Autism spectrum disorder.

Disorders consider neuro-developmental in origin and that have neuro-developmental consequence when occurs in infancy or childhood. This includes:

Autism and Autism spectrum disorder such as Asperger syndrome.

- Intellectual disability (ID) or intellectual and development disability (IDD).
- Fetal alcohol spectrum disorder.
- Traumatic brain injury (including congenital injuries which causes cerebral plasy).[11]
- Motor disorders such as developmental coordination disorders, stereotypic movement disorder.
- Communication, speech and language disorder.
- Genetic disorder such as Fragile- X disorder.
- Down syndrome.
- Schizophrenia.

Neurodevelopmental disorders are associated with widely varying degrees of difficulty which are significant mental, physical, emotional and economical consequence for individual and it turn their family and society also.

1.2. Cause: [12, 13]

The development of brain is tightly regulated and genetically encoded process with clear influence from environment. This suggest that any deviation from this program early in life can result in neurodevelopment disorders and depending on specific timing, might lead to distinct pathology later in life [12]. Because of that there are many causes of neurodevelopmental disorder, which can range from deprivation, genetic and metabolic diseases, immune disorder, infectious diseases, nutritional factor, physical trauma and environmental factors.

Some neurodevelopmental disorders are autism and other pervasive developmental disorders are considering multifactorial syndrome [13].

Various causes of neurodevelopment disorder:

- Immune disorder.
- Infectious disorder.
- Deprivation
- Genetic diseases.
- Metabolic diseases.

In early prenatal stage neurodevelopment start with a complex neurological development which begins with creation of neurons and radial glia and continue to develop in postnatal stage. This process will complete after 3yrs old.

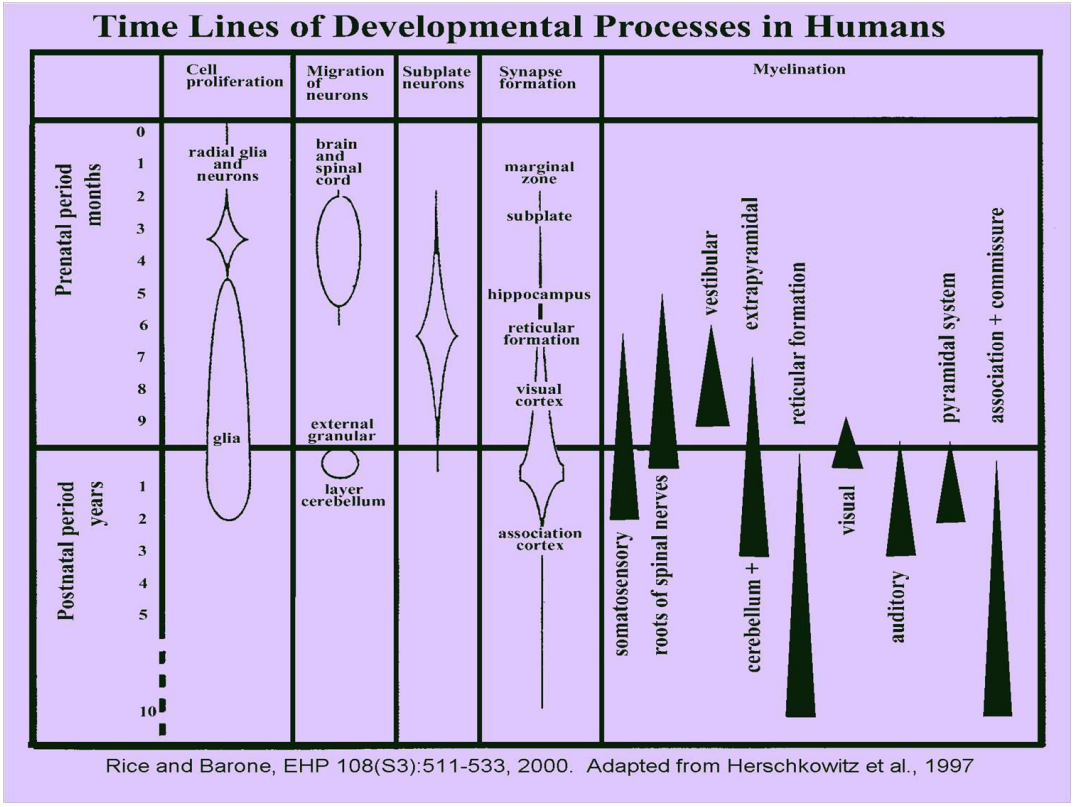
Migration of neurons, which occurs from the second to the six month of gestation and again within the cerebellum postnatal, is a very important and complex process.

Children neurodevelopmental and intellectual disorder [14,15]

Neurodevelopmental Process

- ❖ Abnormalities in maturation may underlie neurodevelopmental disorder.
- ❖ Increased pruning with childhood –onset schizophrenia; decreased with autism.

Fig.1. Neurodevelopment process in Human



)

1 in 6 children in the industrialized countries: [16]

- Cerebral plasy
- Autism
- Decreased IQ(intelligence quotient)
- ADHD (attention deficit hyperactivity disorder)
- Learning disabilities
- Developmental delay

In industrialized countries neuro-developmental disorders occurs commonly. It has been figured that 15% of children's are having learning disability, autism, ADHD, and developmental delay. The prevalence is much higher in aboriginal children. Although some cases are linked to identify the exposure e.g.; fetal alcohol, tobacco smoke, low birth weight and in many cases etiology is not known.

2. REVIEW AND LITERATURE:

2.1 Autism Spectrum Disorder

Autism is a neurodevelopment disorder which is characterized by impaired social interaction, verbal and non-verbal communication and repetitive behavior. First two years of child hood parents can identify the sign of autism[17]. These signs are develop gradually, though some child's with autism is reach their developmental target at normal pace and then degenerate[18]. The investigative criteria require that symptoms became apparent in early childhood, typically before age three[19]

Autism is a highly heritable diseases, researcher suspect both genetic fact as well as environmental fact as causes[20], in rare case autism is strongly related with agent that cause birth defect[21]. Controversies envelop that other proposed environmental causes[22] for example the vaccine hypothesis have been disproven. Autism affects in order processing in the brain by altering how nerve cells and their synapses connect and organizes; how this occurs is not well unstated[23]. In the DSM V it is one of three recognize disorders in the autism spectrum(ASDs), the other two being Asperger syndrome, which lacks delays in cognitive development and verbal communication and pervasive development disorder not otherwise specified , which is diagnosed when full set of criteria for autism or Asperger syndrome are not met[24].

Behavioral intervention can help a child with autism gain self-care, communication and social skills[17]. There is no known cure which has been reported on children who has recovered[25]. Not many children with autism live independently after reaching old age, however some became successful[26]. An autistic culture has developed with some individual seeking a cure and others believing autism should not be treated as disorder, should be accepted as a difference[27].

Internationally autism is estimated to affect 21.7 million people as of 2013 [28]. As of 2010, the number of people affected is expected at about 1-2 per 1000 worldwide. It occurs four to five times more than in boys than girls. About 1.5% of children in the United States are diagnosed with ASD as of 2014, an increase from one in 88 in 2012 [29,30,31]. The rate of autism among adults aged 18years and over in the United Kingdom is 1.1% [32.] The number

of people diagnosed has been increasing radically since the 1980s, partly due to change in diagnostic practice and government - subsidized economic incentives for named diagnoses[33].

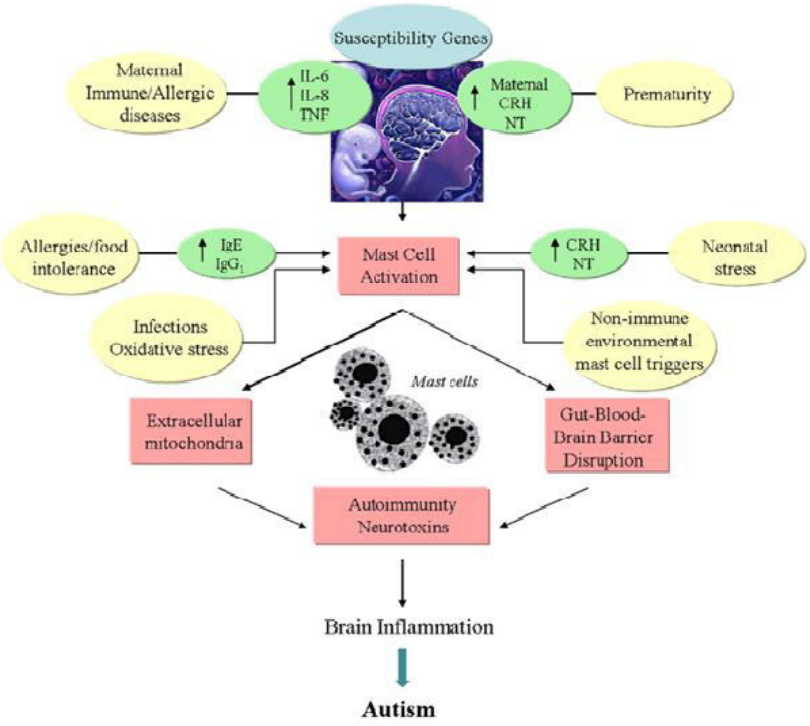
1.2.Characteristic:

Autism is highly variable neurodevelopment disorder[34] that first appears during infancy and childhood and generally follows a stable course without reduction[35]. People with autism may be severely impaired in some respect but normal or even advanced in others[36]. Evident symptoms gradually begin after the age of six months, became established by age two or three years[37], and have a tendency to continue through adulthood, even though often in more muted form[38]. It is illustrious not by a single symptom, but by a characteristic triad of symptoms; impairments in social interaction, impairments in communications and restricted interest and repetitive behavior. Other aspects, such as atypical eating are also common but are not essential for diagnosis[39]. Autism individual symptoms occur in the general population and appear not too associated highly, without a sharp line separating severe from common trials[40].

Characteristic includes;

- **Social development**
- **Communications**
- **Repetitive / restricted behaviour[41]**
  - ❖ Stereotype
  - ❖ Compulsive behaviour
  - ❖ Sameness
  - ❖ Ritualistic behaviour
  - ❖ Self-injury (skin picking, hand biting, eye pocking).
- **Other symptoms:[42]**
  - ▪ Poor motor planning
  - ▪ Poor muscle tone
  - ▪ Toe walking
  - ▪ Sensory abnormalities
  - ▪ Stress
  - ▪ Sensation seeking.

Fig 2: Schematic represents action of autism



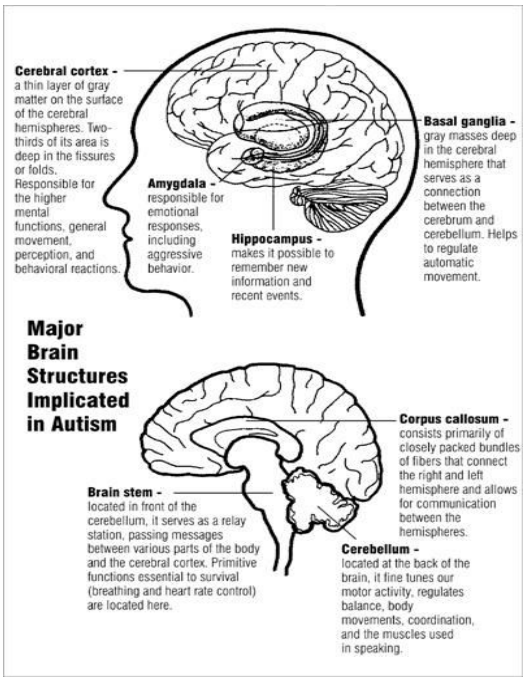
1.3 Pathophysiology Of Autism:

Different scores of other brain disorders such as Parkinson's, autism does not have a apparent unifying mechanism at either the molecular, cellular or system level; it is not known whether autism is a few disorders caused by mutations converging on a few common molecular pathways, or is a large set of disorders with miscellaneous mechanism[34]. Autism appears to result from developmental factors that affect many or all functional brain systems[43], and to disturb the timing of brain development more than a final product[44]. Neuro-anatomical studies connected with teratogens strongly suggest that autism's mechanism includes alteration of brain development rapidly after conception. This variance appears to start a cascade of pathological events in the brain that are radically influenced by environmental factors[45]. Just after birth the brain of children with autism have a tendency to grow faster than usual, followed by normal or moderately slower growth in childhood. It is not known whether early overgrowth occurs in all children with autism. It seems to be most well-known in brain areas underlying the development of higher cognitive specialization.

Hypotheses for the cellular and molecular bases of pathological early overgrowth include the following:

- An overload of neurons that causes local more than connectivity in brain region[46].
- Disturbed neuronal migration during early gestation[47, 48.]
- Unbalanced excitatory-inhibitory networks[48].
- Abnormal formation of synapses and dendrites spines[48].

Fig3: Autism affects on various parts of brain[44].



The immune system is thought to play an important role in autism. Children with autism have been found by researchers to have inflammation of both the peripheral and central immune system as indicated by increased levels of pro-inflammatory cytokines and significant activation of microglia [49, 50, 51]. Biomarkers of abnormal immune function also have been associated with increased impairments in behaviors that are characteristic of the core feature of autism such as deficits in social interactions and communications [50]. Interaction between the immune system and nervous system is said to start during the emergent stage of life and successful neurodevelopment depends on a balanced immune

response. It is through that activation of pregnant mother's immune system such as from environmental toxicants or infection can contribute to causing autism through causing a disruption of brain development [52, 53, 54.] This is supported by recent studies that have found that infection during pregnancy is associated with an increased risk of autism [55, 56].

The relationship of neuro-chemicals to autism is not well understood; several have been investigated, with most of the indication for role of serotonin and of genetic differences in its transfer[23]. The role of metabotropic glutamate receptors (mGluR) in the pathogenesis of Fragile X syndrome, the most ordinary identified genetic cause of autism, has led to interest in the possible implications for future autism research into this pathway[57]. Some data suggest neuronal overgrowth potentially related to an excess in numerous growth hormones[58] or to impaired instruction of growth factor receptors. A few inherent errors of metabolism are associated with autism, but probably account for less than 5% of cases[59].

1.4 Causes:

Many causes of autism have been projected, but understanding of the theory of causation of autism and the autism spectrum disorders is imperfect [60.] Research indicates that genetic factors dominate. The heritability of autism, though, is complex, and it is normally unclear which genes are dependable[61]. In rare cases autism is strongly connected with agents that cause birth defects[62]. Several other causes have been proposed, such as childhood vaccination, but frequent epidemiological studies have shown nonscientific evidence behind any link between vaccinations and autism[63].

Although specific causes of autism spectrum disorders yet have been found, several risk factor has been identified in the research journalism that may contribute to their development. This risk factor includes genetic, prenatal factors, neuro-anatomical abnormalities, and environmental factors. It is possible to identify general risk factors, but much more difficult to identify specific factors. In the current state of knowledge, prediction can only be a universal nature and therefore requires the use of general markers[64].

Genetic risk factors

The report of family and twin studies state that genetic factor play an important role in the etiology of autism and other pervasive development disorders[65]. Studies have consistently found that the occurrence of autism in siblings of autistic children is approximately 15-30 times greater than the rate in the general population[66]. In addition, researchers suggest that there is a much higher concordance rate among monozygotic twins compared to dizygotic twins [67]. It appears that there is no single gene that can account for autism. There are multiple genes involved, each of which is a risk factor for components of the ASD [68, 69 70.]

Prenatal and perinatal risk factors

Numerous prenatal and perinatal complications have been reported as possible risk factors for autism. These risk factors includes maternal gestational diabetes, maternal and paternal age over 30, bleeding after first trimester, use of prescription medication(e.g. velporate) during pregnancy and meconium in amniotic fluid. Whereas research is not conclusive on the relation of these factors to autism, each of these factors has been identified more frequently in autistic children compared to their non-autistic siblings and other usually developing youth [71].

Vaccine controversy:

The most controversial claim concerning autism etiology was the 'vaccine controversy '[72] this speculate, arising from a case of scientific misconduct [73], suggested that autism result from brain damage caused either by (1) measles, mumps, rubella (MMR) vaccine or by (2) thimerosal, a vaccine preservative [74.] No credible scientific support these claims and further indication continues to regulate them, including the observation that the rate of autism continues to climb despite exclusion of thimerosal from routine childhood vaccine [75]. A 2014 meta- analysis examined ten major studies on autism and vaccines connecting 1.25 million children worldwide; it included that neither the MMR vaccine, which has never contained thimerosal [76], nor the vaccine components thimerosal or mercury, lead to the development of ASDs [77].

2.5. History & Nomenclature:

The New Latin word *autimus* (English translation autism) was coined by the Swiss psychiatrist Eugen Bleuler in 1910 as he was defining symptoms of schizophrenia. He derived it from the Greek word *autos* (*autos* meaning"self''), and used it to mean morose self-admiration, referring to autistic withdrawal of the patient to his fantasies, against which any influence from outside becomes an unbearable disturbance [78].

The word *autism* first took its modern sense in 1938 when Has Asperger of the Vienna University Hospital adopted Bleuler's terminology *autistic psychopaths* in a lecture in German about child psychology [79]. Asperger was investigate an ASD now known as Asperger syndrome through for various reasons it was not widely recognize as a separate diagnosis until1981 [80]. Leo Kanner of the Johns Hopkins Hospital first used *autism* in the modern sense in English when he introduced the label *early infantile autism* in a 1943 report of 11 children with striking behavioral similarities. Almost all the characteristics described in Kanner's first paper on the subject notably 'autistic aloneness" and ''insistence on sameness", are still consider as typical of the autistic spectrum disorders. It is not known whether Kanner derived term independently of Asperger[81].

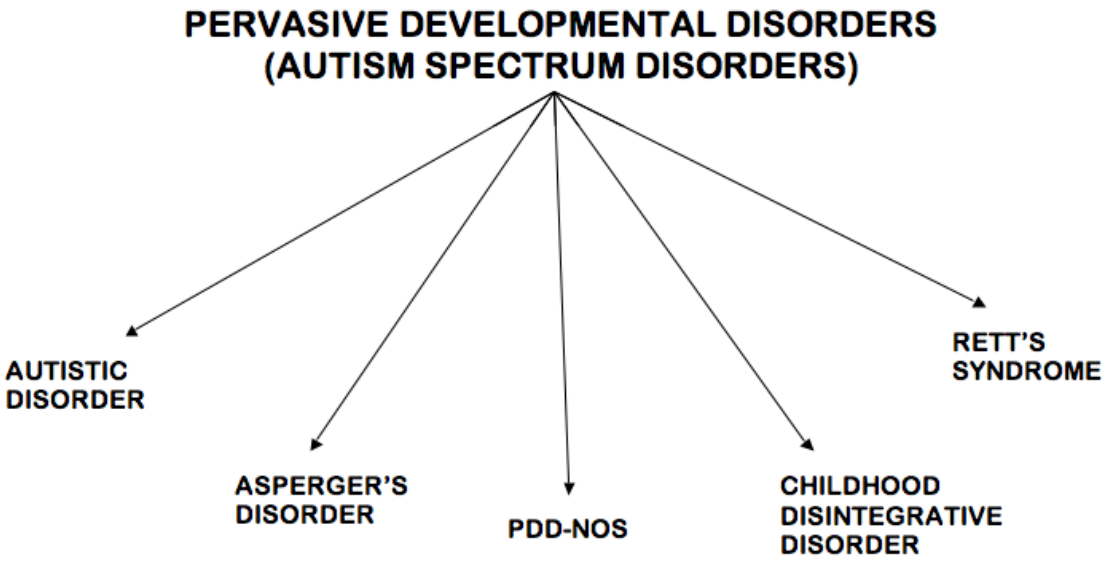
Kenner's reuse of *autism* led to decades of confused terminology like *infantile schizophrenia*, and child psychiatry's focus on maternal deprivation led to misconceptions of autism as an infant's response to'' refrigerator mothers''. Starting in the late 1960s autism was recognized as a separate syndrome by representing that it is lifelong distinguishing it from intellectual disability and schizophrenia and from other developmental disorder, and demonstrating the benefits of involving parents in active programs of therapy[82]. As late as the mid-1970s there was little evidence of a genetic role in autism; now it is thought to be one of the most heritable of all psychiatric conditions[83]. Even though the rise of parent association and the destigmatization of childhood ASD have extremely affected how we view ASD, parents continue to feel social sigma in situations where their child's autistic behavior is perceived negatively by others[84], and many primary care physicians and medical specialists still express some viewpoint consistent with outdated autism research [85].

2.6. Different Types of ASD: [86, 87]

(ASD also known as pervasive developmental disorders)

According to the National Institutes of Health, "The diagnostic category of pervassive developmental disorders (PDD) refers to group of disorders characterized by delays in the development of socialization and communication skills." In fact, that group of disorder is identical with disorders otherwise known as autism spectrum disorders. They include:

- Asperger's Disorder
- Rett's Syndrome
- Childhood Disintegrative Disorder
- PDD-NOS
- Autistic Disorder.



Rett's Syndrome

Rett's syndrome is a genetic disorder that affects only girls. It is the only one of the former autism spectrum disorders that can be diagnosed medically (so far) and as of May 2013, it is no longer included in the Autism Spectrum. Girls with Rett syndrome develop severe symptoms including the characteristic social communication challenges of autism.

PDD-NOS

"Pervasive Developmental Disorder Not Otherwise Specified" is a mouthful of words that are often applied to people on the autism spectrum. It describes individuals who don't fully fit the criteria for other specific diagnoses but are nevertheless autistic. Because there is no easy way to define the symptoms of PDD-NOS, which may range from very mild to very severe, the diagnostic category no longer exists, though a new diagnosis, Social Communication Disorder, may become a similar "catchall" category.

Asperger's Syndrome

Asperger's syndrome is the mildest form of autism, these affects boys three times more than girls. Children with AS became obsessively interested in a single object or topic. They often learn all about their favored subject and discuss it nonstop. Their social skills though are markedly impaired, and they are awkward and uncoordinated.

Asperger's syndrome is mild compared to other ASDs. Also, children with AS frequently have normal to above average intelligence. As a result, some doctors call it "high-functioning autism." As children with AS enter adulthood, though, they are at high risk for anxiety and depression.

Autistic disorder

Children who assemble more rigid criteria for a diagnosis of autism have autistic disorder. They have more severe impairments involving social and language functioning, as well as repetitive behaviors. Often, they also have mental retardation and seizures.

Childhood Disintegrative Disorder

The most severe autistic spectrum disorder, childhood disintegrative disorder (CDD), is also the least common.

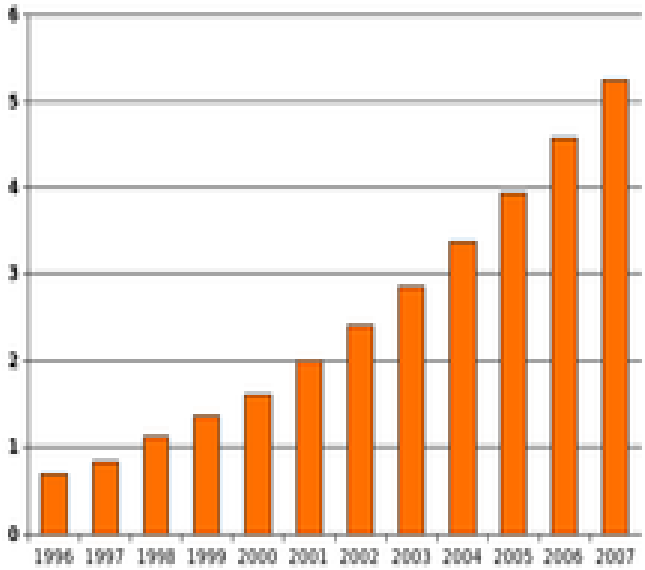
After a period of normal development, usually between ages 2 and 4, a child with CDD rapidly loses multiple areas of function. Social and language skills are lost, as well as intellectual abilities. Often, the child develops a seizure disorder. Children with childhood disintegrative disorder are severely impaired and don't recover their lost function.

Fewer than two children per 100,000 with an autistic spectrum disorder meet criteria for childhood disintegrative disorder. Boys are affected by CDD more often than girls.

2.7. Epidemiology: [88-90]

In recent review have a tendency to estimate a prevalence of 1-2 per 1000 for autism and close to 6 per 1000 for ASD and 11 per 1000 children in the United States for ASD as of 2008 because of inadequate data, these numbers may underestimate ASDs true rate. Worldwide, autism affects an estimated 21.7 million people as of 2013, while Asperger syndrome affects a further 31.1 million. In 2012, the NHS estimated that the overall prevalence of autism among adults aged 18 years and over in the UK was 1.1%. Rates of PDD-NOS's have been estimated at 3.7 per 1000 Asperger syndrome at approximately 0.6 per 1000 and childhood disintegrative disorder at 0.02 per 1000. CDC's mainly recent estimation is that 1 out of every 68 children, or 14.7 per 1000, has ASD as of 2010

Graph 1: **Reports of autism cases per 1,000 children grew dramatically in the U.S. from 1996 to 2007**



Several other conditions are common in children with autism. They include:

Genetic Disorder: [91,92]

Around 10-15% autism cases have a particular single-gene condition, chromosomes abnormality or other genetic syndrome, and ASD is associated with various genetic disorders.

Intellectual Disability[93, 94,95]

Several studies have been reported 25-70 percentage of autistic individuals who also meet criteria for intellectual disability, a wide variation illustrating the difficulty of assessing autistic intelligence. Intellectual disability is much weaker compared with PDD-NOS association.

Anxiety Disorders[96]

In ASD anxiety disorder is common in children, there is no firm data but studies have been reported prevalence ranging from 11-84%. Most of the anxiety disorders have symptoms that are better explained by ASD or are hard to distinguish from ASD's symptoms.

Epilepsy[97]:Variations in risk of epilepsy due to age, cognitive level, and type of language disorder.

Metabolic defects such as phenylketonuria are associated with autistic symptoms.

Minor physical anomalies[98] are significantly increased in the autistic population.

Preempted diagnosis [99] Even though the DSM-IV rules out concurrent diagnosis of many others conditions along with autism, the full criteria for Attention deficit hyperactivity disorder (ADHD) Tourette syndrome, and other of these conditions are frequently present and these comorbid diagnoses are increasingly accepted.

Sleep problems[100] affect about two-thirds of individuals with ASD at some point in childhood. The most commonly include symptoms of insomnia such as difficulty in falling asleep, frequent nocturnal awakenings, and early morning awakenings.

2.8. Screening And Diagnosis: [101]

Diagnosing of autism spectrum disorder (ASD) is difficult, since there is no medical test, like a blood test, to diagnose the disorders. Doctors look at the child's behavior and development to make a diagnosis.

Sometimes ASD can be detected at 18 months or younger. At an age 2, a diagnosis by an experienced professional can be considered very dependable. Though, many children do not receive a final diagnosis until much older. This delay means that children with an ASD might not get the help they need.

Diagnosing an ASD takes two steps:

- Developmental Screening
- Comprehensive Diagnostic Evaluation
-

Developmental Screening

Developmental screening is a small test to inform about children is learning basic skills when they should, or if they might have delays. During developmental screening the physician may ask the parents about the child's behavior, learning ability and movements etc. a delay of this area could be a sign of a problem.

All children should be screened for developmental delays and disabilities during regular well-child physician visits at:

- 9 months
- 18 months
- 24 or 30 months
- Additional screening might be needed if a child is at high risk for developmental problems due to preterm birth, low birth weight or other reasons.
- In addition, all children should be screened specifically for ASD during regular well-child doctor visits at:

- 18 months
- 24 months
- Additional screening might be needed if a child is at high risk for ASD (e.g., having a sister, brother or other family member with an ASD) or if behaviors sometimes associated with ASD are present

If any signs of a problem found by physician, then a comprehensive diagnostic evaluation is needed.

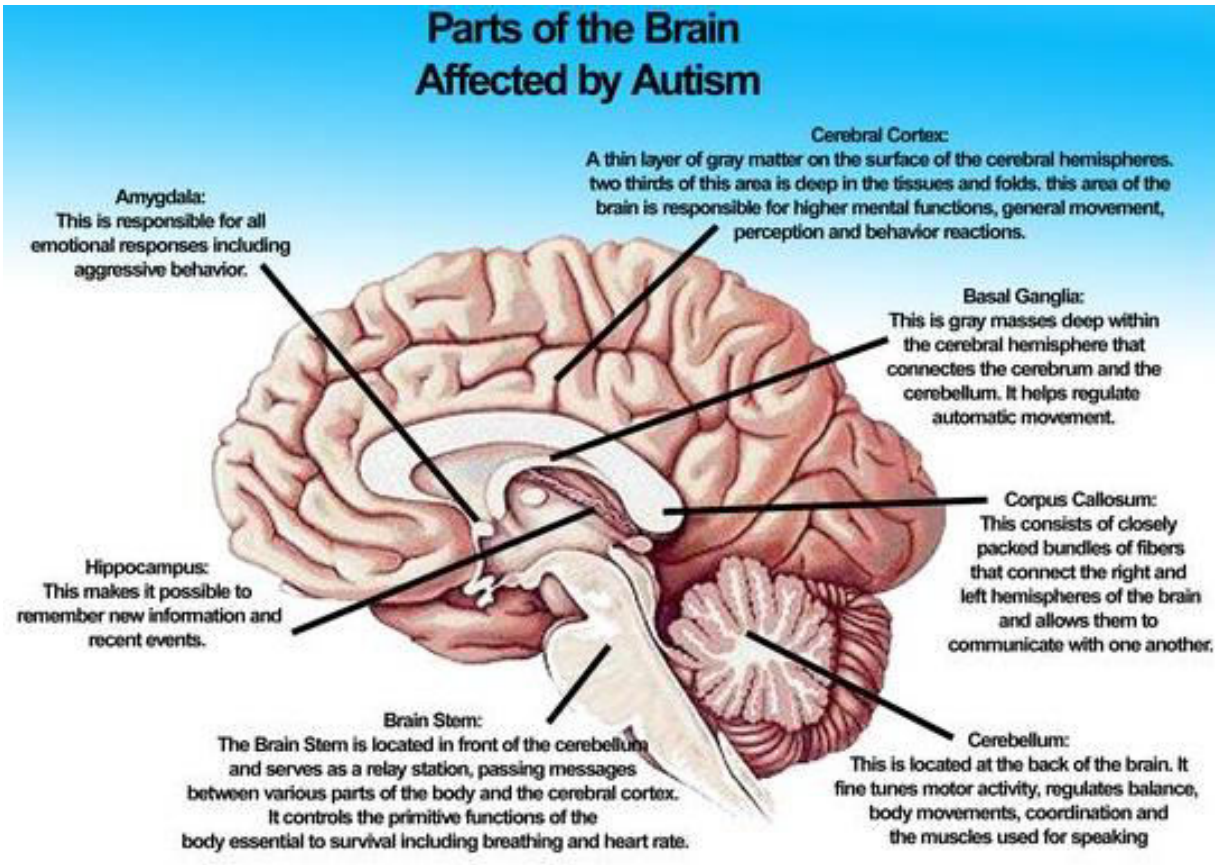
Comprehensive Diagnostic Evaluation

Comprehensive evaluation is a second step of diagnosis. This review may include looking at the child's behavior and development and interview the parents. It may also include a hearing and vision screening, genetic testing, neurological testing, and other medical testing.

In several cases, the primary care might decide to refer the child and family to a specialist for further assessment and diagnosis. Specialists who can perform evaluation include:

- Developmental Pediatricians (doctors who have special training in child development and children with special needs)
- Child Neurologists (doctors who work on the brain, spine, and nerves)
- Child Psychologists or Psychiatrists (doctors who know about the human mind)

Fig4: Parts of brain affected by Autism



2.9. Treatment [102-107]

Different types of treatments are accessible to treat ASD. For example: facilitated communication, auditory training, vitamin therapy, anti-yeast therapy, physical therapy, occupational therapy, and sensory integration.

The treatments can be generally categorized as follows:

Curriculum development

Dietary Approaches

Pharmacotherapy

Complementary and Alternative Medicine

Curriculum development

Based on the child's unique pattern of strengths and weaknesses, educational interventions must be individualized. Comprehensive educational plans for a child with ASD include the following areas: social skills, communication, cognitive skills, and adaptive behaviors', sensory and motor development.

Dietary Approaches

Removing certain types of foods especially those are rich in gluten and casein from a child's diet and using vitamins like B_{12} or mineral supplements improve the behaviors' being aggressive. Dietary treatments are based on food allergies or lack of vitamins and minerals that cause symptoms of ASD.

Complementary and Alternative Treatments

To relieve the symptoms of ASDs, some health care professionals provide them complementary and alternative medicine (CAM). It includes special diets, biological (e.g., secreting), chelation (to remove heavy metals like lead from the body), or body-based

systems (like deep pressure, Acupuncture), Vitamins like B_{12}, Folic acid, Omega-3, and Melatonin to improve sleep cycle.

Pharmacotherapy

There are **no medications available that can cure ASD or treat the core symptoms**. However, there are few medications to help people with ASD function better. Medication may help in managing inability to focus, high energy levels, depression or seizures. But all medications may not affect all autistic individuals in the same way.

It is important that there are **no medications** still to target social and language impairment of autism specifically. Medications usually used to control the symptoms are:

- **Risperidone and aripiprazole** - approved by the FDA for the treatment of irritability associated with ASD.[108]
  - **Risperidone** is a dopamine D2 or serotonin (5HT) receptor antagonist and have powerful antipsychotic properties. It has a greater risk of weight gain.
  - **Aripiprazole** is also an antipsychotic agent with high affinity towards serotonin 5-HT1A and 5-HT2A receptors and dopamine D2 and D3 receptors. It has a relatively lower risk for weight gain.
- **Citalopram,** an antidepressant drug has no effect against ASD instead showed serious adverse effects like hyperactivity, impulsiveness, stereotypy and decreased concentration[109].
- **Fluoxetine** is also an antidepressant drug but it has been related with improvements in stereotypy, irritability, lethargy, and inappropriate speech and improved functioning of behaviors in persons with ASD or mental retardation have been observed.[110,111]
- **Fluvoxamine** is used for reducing repetitive thoughts and behavior, aggression, and in improvement in some aspects of social relatedness, especially language usage. Side effects like Mild sedation and nausea are reported.[112]
- **Venlafaxine** is a potent inhibitor of 5-HT, norepinephrine and to a lesser extent, dopamine reuptake. It improves repetitive behaviors, hyperactivity, communication, inattention[113]

- **Lithium or other mood stabilizers** are used rarely in treating cyclic irritability and explosiveness associated with ASD.[114]
- **Stimulants** showed short attention span, impulsivity, and hyperactivity in some autistic individuals; however they may worsen the stereotypies and behavior in some individuals with ASD[115].
- The hyper arousal behaviors such as stereotypic movements, hyperactivity, and self-stimulation evident with many individuals are treated with **clonidine**, a α_2 adrenergic receptor agonist. Side effects like sedation and fatigue were not noted but develop tolerance to sedatives but not the therapeutic effects of clonidine.[116,117]
- **Propranolol** (beta blockers) is used in the management of aggression, agitation and anxiety. Since the half-life of this drug is short (~ 4 hrs), frequent administration is necessary. Hypotension and bradycardia occurs as side effects.[118]

2.10. Experimental Animal Models For Autism Spectrum Disorder

Mechanical induced ASD

ASD model on macaque monkeys [119].

This is the most successful model developed till date. It involves the removal of bilateral removal of the medial temporal lobe of young macaque monkeys. The animal shows a svariety of social and emotional changes like human including social isolation. It also demonstrates other autistic symptoms such as stereotypical behaviors.

Early lesions of cerebellum, amygdala and medial prefrontal cortex also induce ASD

Chemical induced ASD

***In utero* exposure to thalidomide and valproic acid [120,121]**

In this model rats are prenatally exposed to terotogens (like thalidomide and valproic acid). In utero exposure to teratogens in the 1st trimester (12th day of gestation) of rat affects the cranial nerve nuclei and motor nuclei of foetus resembling neuropathology of Autism spectrum disorder in human.

Propionic acid induced ASD

Propionic acid is produced by gut bacteria such as clostridia and propionic bacteria. Intracerebro ventricular (*icv*) infusion of propionic acid produces ASD symptoms in rodents.

Infection induced ASD

Neonatal Borna Disease virus (BDV) infection [122]

Many of the features of pathogenesis, expression of neuro-anatomical and behavioral disease in neonatally BDV infected rats resemble ASD. Persistent infection of BDV on brain of neonate rat affects hippocampus which produces deficits in emotional and cognitive domains. It also causes metabolic alterations in selective neuronal populations without a

gross injury. BDV infection allows us to study the brain-behavioral relationship from a developmental standpoint.

2.11. INTRACEREBRO VENTRICULAR INFUSION OF PROPIONIC ACID (PPA): ASD EXPERIMENTAL ANIMAL MODEL[123]

Introduction

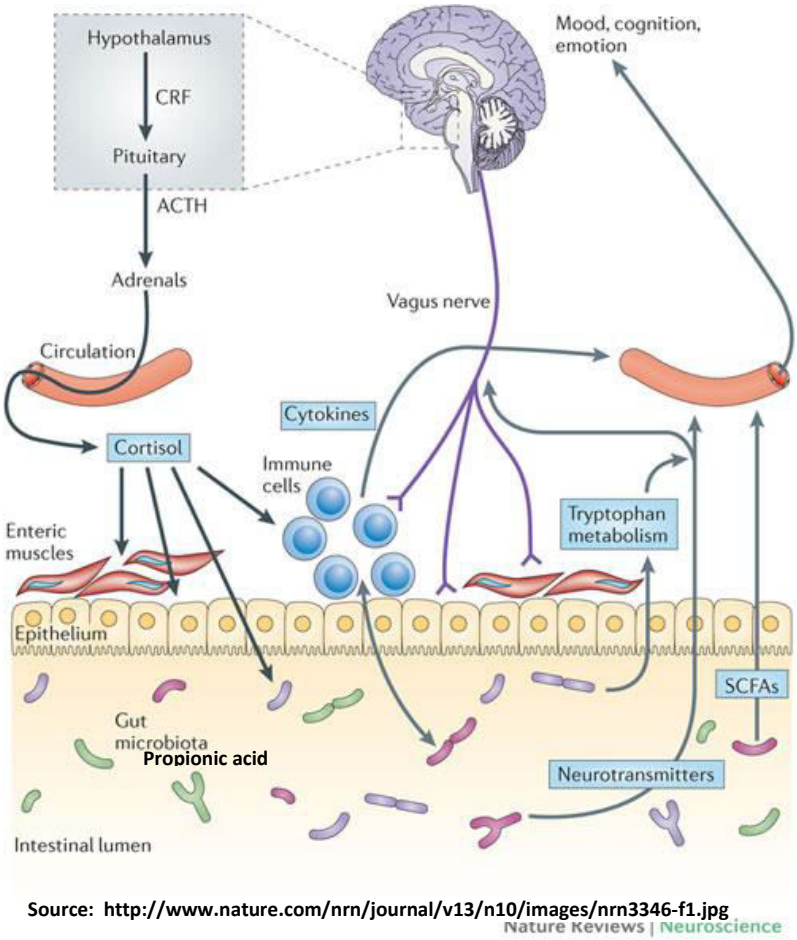
An emerging possibility is that ASD is a systematic encephalopathic condition involving digestive, immune and metabolic dysfunction exacerbated by environmental triggers. ASD patients have high levels of *Clostridia or Bacterioidetes* in the gut, which produce propionic acid (PPA) and other fatty acids by anaerobic fermentation of dietary carbohydrates and some amino acids.

Propionic acid (PPA) is a short chain fatty acid that is endogenous to the human body as both an intermediary of fatty acid metabolism and a metabolic end product of enteric gut bacteria, *Clostridia and Propionibacteria*, a common food preservative in refined wheat and dairy products. PPA, being a weak acid, exists in both aqueous and lipid soluble forms and can readily enter the systemic and CNS environments (blood–brain barrier) both via monocarboxylate transporters and can cause health-related issues if the individual is unable to metabolize short-chain fatty acids adequately. PPA and other short-chain fatty acids (i.e. butyrate and acetate), affect diverse physiological processes such as neurotransmitter synthesis and release, cell signaling, lipid metabolism, immune functions, mitochondrial function, gap junctional gatin, and modulation of gene expression through DNA methylation and histone acetylation.

Mechanism of action

PPA is known to increase intracellular neuronal and glial acidification. It also increases calcium proportions, thereby affects neurotransmitter release including serotonin, dopamine, glutamate, and norepinepherine each of which play a role in elicitation of abnormal behavior and locomotor activity. PPA has been shown to increase glutamatergic transmission, leading to excitability in brain regions linked to locomotor activity.

Fig 5: Communication pathway between propionic acid of gut microbiota and neurotransmitters and inflammatory mediators



Phospholipids are the major structural components of neuronal and other cellular membranes, and include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM). All of these phospholipid classes were observed to have altered molecular species distribution following PPA infusion. The pathological consequences of disturbances in phospholipid metabolism could include alterations in signal transduction involving the generation of second messengers derived from arachidonic and docosahexaenoic. The observation that PPA infusion increased the proportions of brain PI and PC molecular species containing arachidonic acid and decreased the proportions of PS and PE molecular species with docosahexaenoic acid, suggests that PPA could influence the innate neuroinflammatory process observed in ASD. Eicosanoids are inflammatory mediators that induce the formation

of proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin 1 (IL-1), and interleukin 6 (IL-6). The increased accumulation of brain molecular species with the eicosanoids precursor (arachidonic acid) and the decreased proportions of the molecular species, containing the docosanoids precursor (docosahexaenoic acid) increase neuroinflammation. In both brain and blood membranes, PPA infusions alter the PC and PE composition (both diacyl and plasmalogen forms). The alterations observed in plasmalogen molecular species in brain and blood polyunsaturated PE and PC molecular species, increases oxidation following PPA infusions.

PPA is known to reversibly inhibit mitochondrial function via the production of the cytotoxin propionyl Coenzyme A and through the sequestration of carnitine, leading to impairments in fatty acid metabolism. Both of these effects could lead to a diffuse encephalopathic process due to an intercellular accumulation of PPA and other fatty acids, with resultant increases in oxidative stress. Oxidative damage of lipids has been suggested to play a part in the pathogenesis of many neurological diseases. Oxidative damage has been shown to uncouple the gap junctions in astrocytes. Arachidonic and docosahexaenoic acids which are very susceptible to oxidative damage have been shown to modulate the coupling capacity of gap junctions.

2.12. Literature Review Of Autism Spectrum Disorder

1. Screening And Diagnosis Of Autism.[124]

Filipek, P.A et.al has discussed about the current criteria for identifying and diagnosing autism spectrum disorders and the experimental evidence in which they are based. The authors also provide recommendations for a dual process approach of routine developmental observation to identify children at-risk for any developmental disorders and those exclusively at-risk for autism spectrum disorders and, secondly, to identify aspects individual autism from other developmental disorders.

2. Autism: An Emerging 'Neuroimmune Disorder' In Search Of Therapy[125]

Duraisamy Kempuraj *et al* have reviewed epidemiology, pathogenesis and treatment of ASD and suggested increased oxidative stress and immune dysregulation are present in ASDs. They also suggested that Mast cell activation may contribute to gut–blood–brain barrier disruption and brain inflammation.

3. Hearing-impaired autistic children[126]

Jure, R,et al have reviewed audio-logical information for a group of 46 children diagnosed with both austism and hearing loss. They concluded that while the severity of the autism diagnosis was related to the cognitive dysfunction, it was not linked to the severity of the hearing loss. Moreover, the review noted that almost 25% of the children remained undiagnosed with autism for a period of at least 4 years.

4. Alterations of GABAergic Signaling in Autism Spectrum Disorders[127]

Enrico Cherubini and Rocco Pizzarelli summarized that ASDs are associated with single mutations in genes encoding for neuroligin-neurexin families. These molecules regulate trans-synaptic signaling contributing to maintain a proper excitatory/inhibitory (E/I) balance at the network level. GABA in adult life has been shown to depolarize and excite targeted cell through an outwardly directed flux of chloride. In early development of life, GABA has a functional role in building up and refining neuronal circuits and the molecular mechanisms regulating the excitatory/Inhibitory, E/I balance. A dysfunction of the

GABAergic signaling early in development leads to a severe E/I unbalance in neuronal circuits, a condition that may account for some of the behavioral deficits observed in ASD patients.

5. Peripheral auditory asymmetry in infantile autism.[128]

Khalfa, S.,et al. explored the contra lateral suppression of otoacoustic emissions in individuals with autism and age- and gender-matched typically-developing individuals. Inhibition of otoacoustic emissions is hypothesized to result from activity of the efferent medial-olivo-cochlear pathway and is thought to be concerned in the filtering of background noise. Results of the study demonstrated differing patterns of irregularity between groups as well as an age-related decrease in otoacoustic emission amplitude among autistic individuals. The authors conclude that the findings may suggest involvement of higher-level processes as well as an age-related decrease in outer hair cell function among autistic individuals.

6. Parental Self-perception in the Autistic Spectrum Disorder Literature: a systematic Mixed Studies Review.[129]

Kirsten K Frantzen et al. Illustrates that self-efficacy can be merged into competence and the emergence of the coherence construct. Competence, control, and coherence are vitally important core concepts for understanding parents of children with autism. A unified nomenclature based upon the overarching concept of self-perception would organize the most pivotal constructs much more clearly and reduce redundancies. Specifically, the concept of "parental self-perception" is the most promising candidate for this super ordinate concept.

7. Melatonin In Autism Spectrum Disorders: A Systematic Review And Meta-Analysis [130]

Daniel A Rossignol and Richard E Frye investigated melatonin-related findings in autism spectrum disorders (ASD). They observed abnormality in melatonin and melatonin metabolite. The physiological levels of melatonin and / or melatonin derivatives were found to be below average. Gene abnormalities could contribute to decreased melatonin production or adversely affect melatonin receptor function in a small percentage of children with ASD. Clinical study reported improved daytime behavior with melatonin use

8. Brain Imaging Increases Our Understanding of Developmental Coordination Disorder: a Review of Literature and Future Directions [131]

Meisan Brown-Lum1 &Jill G. Zwicker suggested that children with developmental coordination disorder (DCD) activate different regions of the brain during functional tasks and show differences in white matter microstructure compared to typically developing children. The emerging neuro- imaging data will help clarify the possible underlying mechanisms in relation to impaired motor function at the behavioural level in children with DCD.

9. Advances in the Research of Melatonin in Autistic Spectrum Disorder: Literature Review and New Perspective [132]

Sylvie Tordjman et.al first reviewed the studies on melatonin levels and the treatment studies of melatonin in autistic disorder. After that they discussed about the relationship between melatonin and autistic behavioural impairments with regard to social communications (verbal, non-verbal communications, social interaction), and repetitive behaviours or interests with difficulties adapting necessary to established potential therapeutic efficacy of melatonin for social communication impairments and stereotyped behaviours or interests.

10. Brain Serotonin And Dopamine Transporter Bindings In Adults With High Functioning Autism [133]

Kazuhiko Nakamura *et al* determined the occurrence of changes in the binding of serotonin and dopamine transporters, which are highly selective markers for their respective neuronal systems. They observed that Serotonin transporter binding was significantly lower throughout the brain in autistic Individuals. Specifically, the reduction in the anterior and posterior cingulated cortices was associated with the impairment of social cognition in the autistic subjects. Significant correlation was also found between repetitive and/or obsessive behavior and interests and the reduction of serotonin transporter binding in the thalamus. In contrast, the dopamine transporter binding was significantly higher in the orbito-frontal cortex of the autistic group. In the orbitofrontal cortex, the dopamine transporter binding was

significantly inversely correlated with serotonin transporter binding. The present findings indicate that the gross abnormalities in these neurotransmitter systems may underpin the neurophysiologic mechanism of autism.

11. Neuroimmunology of Autism Spectrum Disorder[134]

David Marc & Kelly Olson summarized the role of cytokines in the patho-physiology of autism has been researched suggesting a relationship with altered blood-brain barrier permeability and subsequent neuro-inflammation. Cytokine recruitment to the CNS may result in altered neurotransmitter signaling and the behavioral manifestation of autism symptoms. Other immune mediated events such as changes in the number and activity of natural killer cells, macrophages, immune-globulins, and glutathione may contribute to altered neuronal signaling and neurotransmitter imbalances.

12. Autism And Immunity: Revisited Study.[135]

Kempuraj DJ *et al* had a consistent finding in autistic children that a high number of mast cells and a high level of serotonin were even found at elevated concentrations in the urine of autistic patients. In addition, a dysfunction of gastrointestinal and immunological symptoms is noted in autistic children. It was suggested that an increase of cytokines/chemokines produced by mast cells at an early age may play an important role.

13. Effects Of The Enteric Bacterial Metabolic Product Propionic Acid On Object-Directed Behavior, Social Behavior, Cognition, And Neuro-Inflammation In Adolescent Rats: Relevance To Autism Spectrum Disorder.[136]

Donald P, Derrick F. Mac Fabe *et al* administered propionic acid (PPA), a short chain fatty acid that is used as a food preservative and also is a metabolic end-product of enteric bacteria in the gut, to adolescent (41±4 days) male rats in a study of restricted/repetitive behavior, social behavior, and cognition. They evaluated the effects of PPA in young rodents. PPA (1µl of 0.26M solution) was administered intracerebro ventricularly prior to each behavioral test. Rats treated with PPA displayed restricted behavioral interest to a specific object among a group of objects, impaired social behavior.

14. Increased Serum Levels Of Glutamate In Adult Patients With Autism.[137]

Shinohe A *et al* studied whether amino acids (glutamate, glutamine, glycine, D-serine, and L-serine) related to glutamatergic neurotransmission is altered in serum of adult patients with autism. Serum levels of glutamate in the patients with autism were significantly higher than those of normal controls. In contrast, serum levels of other amino acids (glutamine, glycine, d-serine, l-serine) in the patients with autism did not differ from those of normal controls. The study suggested that an abnormality in glutamatergic neurotransmission may play a role in the pathophysiology of autism.

15. Oxidative Stress Markers in Children with Autism Spectrum Disorders [138]

María Elena González-Fraguela *et al* quantified the activity of the antioxidant enzyme catalase (CAT), glutathione concentration (GSH) and markers of damage to biomolecules, *malonyldialdehyde* (MDA) and 8–hydroxy-2deoxyguanosine (8OHdG) in peripheral blood samples of ASD affected children to evaluate redox status. The study suggested that the total GSH content in autistic patients was significantly lower compared with the control group. Higher serum CAT, MDA and 8OHdG levels were found in children with autism compared with controls. The study supported the notion that oxidative stress is associated with autism.

3. PLANT PROFILE

Introduction

Plants are the most important source of medicine in the world and writing indicates their medicinal uses as old as 4000-500 B.C. In china first start use of herbal preparations as medicine. In –India Rig-Veda (written between 3500-1600) having earliest source of plant as medicine, and after that the properties and therapeutically uses are recorded in Ayurveda[139]. World Health Organization has listed 20,000 species of medicinal plants which are used in universal[140].

According to WHO more than 80% of World population relies from primary health care by using traditional herbal medicines[141]. Various drugs & chemicals are obtain from various parts of plants and continue to be possible sources of new drugs and chemicals [143, 144]. *Withania somnifera* (L) Dunal is an important medicinal plant and used in Ayurvedic medicines for the treatment of many diseases [145] .

In Auyrveda it is known as Rasayana' because it promote longevity, arrest ageing process, increase ability of individual to resist adverse ecological conditions[146.] The steroidal lactones (withanolides) obtained from its roots are mainly responsible for its therapeutic activities and general health maintenances like immune-modulation, combinating infectious agent, anti-cancer, anti-epileptic, anti-ageing, antioxidant, hypoglycemic, memory enhancer and in common an effective adaptogen.

History and Nomenclature [146]:

Withania somnifera (L) Dunal commonly known as" Ashwagandha"," Asgandh ", and " Winter cherry" belongs to family *Solanaceae* and widely distributed in warmer parts of the world. Genus Withania comprises 23 species including W. somnifera (L), Dunal and W.coagulans (L) Dunal having high therapeutic value which are used as "Rasavana" in Ayurvedic formulations.

Ashwagandha attains the special name because its roots smells like horse ("Ashwa '') and believe to provide power like horse when consumed.(**Ashwa** means

horse. **Gandha** means fragrance. It indicates the property-the strength and sexual vitality of the horse.)

In Vedas it is described as herbal tonic and health food & considered as "Indian Ginseng" because of its ginseng like health promoting effects. Ashwagandha improves energy and also memory by enhancing the brain and nervous function; shows anxiolytic effects, has hepatoprotective property, raises hemoglobin level and red blood cell count, improve the cell-mediated immunity; promotes strength and vitality along with positive sexual life and reproductive stability and act as a powerful adaptogen.

Fig 6: Leaves and root of *Withania somnifera*

Botanical Classification [147-149]

Kingdom : Plantae

Division : Angiosperms

Class : Dicotiledoneae

Order : Tubiflorae

Family : Solanaceae

Genus : *Withania*

Species : *somnifera*

Vernacular Name

Arabic : Kaknaj-e Hindi

Bengali : Ashvaganda , Asvagandha

English : Winter cherry

Gujarati : Asan, Asana, Asoda, Asunda,

Malayalam : Amukkiram, Pevetti

Marathi : Askandha, Kanchuki, Tilli

Odiya : Asugandha

Persian : Asgandh Nagaori, Kaknaj-e- Hindi

Sanskrit : Ashvagandha, Ash vakandika, Gandhapatri, Palashaparni

Tamil : Amukkira, Asubam, Asubagandhi

Telugu : Penneru, Asvagandhi, Dommadolu, Pennerugadda

Urdu : Asgand, Asgand Nagori.

Common Names

Winter cherry (Eng.); Bitterapelliefie , Geneesblaarbossie, koorshout (Afr.); Bofepha (Sotho); Ubuvuma (xhosa); Ubuvimbha (Zulu); Ashwagandha (Hindi).

Geographical Distribution [146]

Withania somnifera is extensively circulated around the world from Southern Mediterranean region to the Canary Island and from South to East Africa; Jordan, Egypt, Sudan, Iran, Afghanistan, and Pakistan. In India the plant is growing wild in the North-Western regions extend to the mountainous region of Punjab, Himachal Pradesh, and Jammu up to an altitude of 1500m.

Description[146]

W. somnifera is an erect green, branched / unbranched herb with height up to 1.25m. The aerial parts like stem, leaves an calyx are covered with fine hairy tomentum. Its branches are rounded; leaves are simple, ovate, entire, shiny smooth and opposite; flowers are inconspicuous, greenish or yellow, in axillary umbellate cymes, bisexual; fruits is a berry in persistent calyx and seeds are small, flat, yellow, very light. During June-July seeds used to sown and the plants are favor sunny situation. It is susceptible to bacterial, fungal, viruses, and pest's infections.

Phytochemical Constituents [150]

The main constituents of Ashwagandha are alkaloids and steroidal lactones. Among the various alkaloids, withanine is the main constituent. The other alkaloids are somniferine, , somniferinine, withananine, pseudo-withanine, tropine, pseudo-tropine,choline, anaferine and anahydrine.

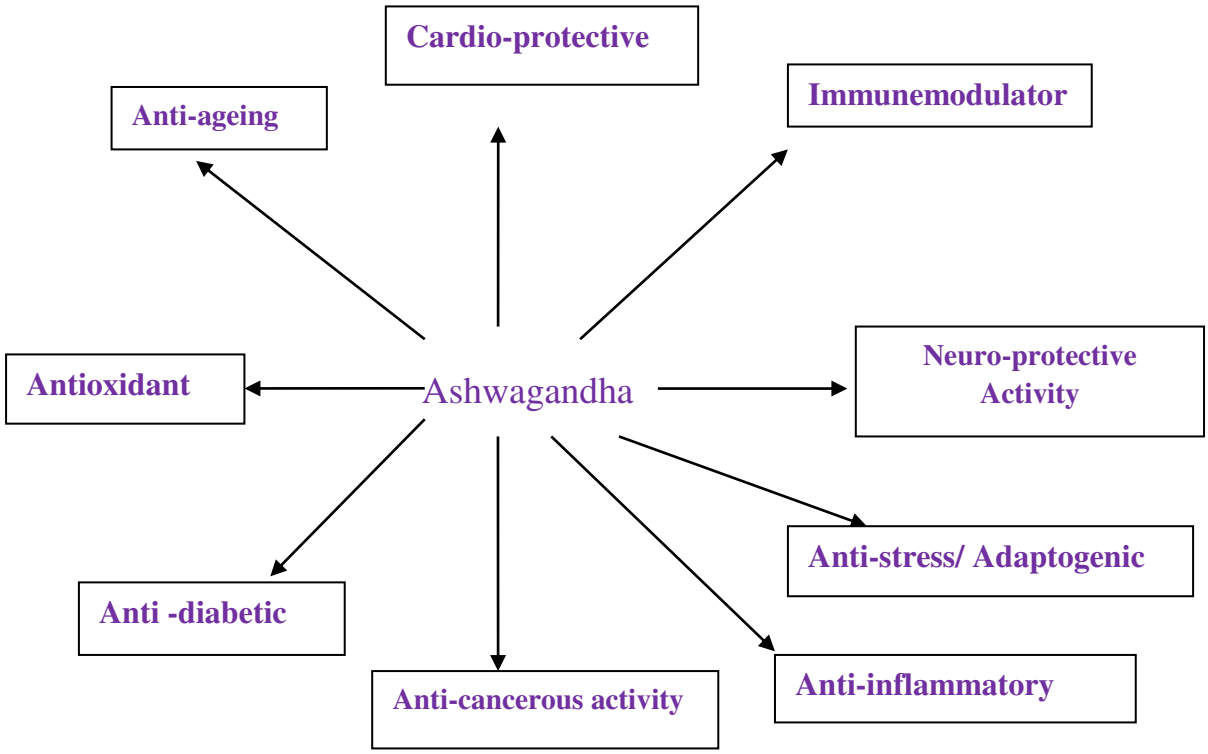
The leaves contain steroidal lactones, which are commonly called as "withanolides". The withanolides have C_{28} steroidal nucleus with C_9 side chain, having six membered lactone rings

Pharmacological and Bioactivities [146]

In Ayurvedic medicine Ashwagandha is widely used, and is an important ingredient prescribed for a variety of musculoskeletal conditions like arthritis, rheumatism and also used as general tonic to increase energy, overall health and longevity.

For investigation of Ashwagandha property many pharmacological studies have been conducted such as antioxidant, anti-ageing, immunemodulatory, cardio-protective, neuro-protective etc. It is most important plants in Indian pharmacopeia and World pharmacopeia.

Fig7: Pharmacological activities of *Withania somnifera*



Traditional uses: [150]

Ashwagandha has sedative and hypnotic effects. It has hypotensive, respiratory, stimulant action along with bradycardia. It is an immunemodulatory agent. It acts as mood stabilizer revives mind and body.

Traditionally it has been used in the treatment of rheumatism, gout, hypertension, nervine and skin diseases. This drug prevents bony degenerative changes in arthritis conditions. It has been widely used as sex stimulant and rejuvenator and is considered as strength & vigor promoting drug especially in geriatric cases.

The leaf extracts shows action against *Staphylococcus aureus* and Ranikhet virus.

3.1. Review and literature of *Withania somnifera*:

1. *Withania somnifera* Improves Semen Quality [151]

Abbas Ali Mahdi et al has been carried out to understand the role of stress in male infertility and to rest the ability of *W .somnifera* to combat stress and treat male fertility. Various biochemical and stress parameters before and after treatment suggested a definite role of stress in male infertility and the ability of *W. somnifera* to treat stress- related infertility. Treatment resulted in a decrease in stress, improved the level of anti-oxidants and improved overall semen quality in a significant number of individuals. The treatment resulted in pregnancy in the partners of 14% of the patients.

2. Adaptogenic Activity Of The Roots *Withania Somnifera* Dun [152]

B. Singh et.al was evaluated for putative anti-stress activity against a sequence tests to delineate the activity of this fraction. The extract of Withania somnifera root was also used to compare the results. A preliminary acute toxicity study in mice showed a good margin of safety with a high therapeutic index.

3. Anticancer activities of Withania somnifera [153]

B. Yadav et.al evaluated *in vitro* cytotoxicity in 50% ethanol extract of root, stem and leaves of *Withania somnifera* against five human cancer cell lines of four different tissues i.e. PC-3, DU-145(prostrate), HCT-15 (colon), A-549 (lung) and IMR-32 (neuroblastoma). This investigation is the first report of the anticancer activity in various parts of *Withania somnifera* cultivated in fly ash amended soil.

4. Immunomodulatory activity of *Withania somnifera* [154]

Girija Kuttan et al. designed that administration of an extract from the powdered root of the plant *Withania somnifera* was found to stimulate immunological activity in mice. Treatment with five doses of *Withania somnifera* root extracts (20mg/dose/animal:i.p.) was found to enhance the total WBC count on 10^{th} day. Bone marrow cellularity as well as α-esterase positive cell number also increased significantly after the administration of *Withania*

somnifera extract. The result proves the immunemodulatory activity of *Withania somnifera* extract, which is known immune-modulator in indigenous medicine.

5. Cardio-protective Effect of *Withania somnifera.*[155]

Ipseeta Mohanty et al. designed to evaluate the cardio-protective potential of hydro-alcoholic extract of *Withania somnifera* the basis of haemodynamic, histopathological and biochemical parameters in the isoprenaline induced myocardial necrosis in rats and to compare with vitamin E, a known cardio-protective antioxidant. Among the different doses studied, *Withania somnifera* at 50mg/kg dose produced maximum cardio-protective effects.

6. Hypoglycemic activity of *Withania somnifera* [156]

Jonathan Gorelick et.al evaluated that in India Ashwagandha is used traditionally to treat many medical problems including diabetes and has verified therapeutic activity in various animal models as well as in diabetic patients. While much of *W. somnifera* therapeutic activity is attributed to Withanolides, their role in the anti-diabetic activity of *W. somnifera* has not been adequately studied. This study evaluated the anti-diabetic activity of *W. somnifera* extract and purified Withanolides as well as the effect of various elicitors on this activity.

7. Pharmacological profile of *Withania somnifera* (Ashwagandha) [157]

Kaur Narinderpal et al. evaluated ashwagandha possesses antioxidant, anxiolytic, adaptogen, memory enhancing, antiparkinsonian, antivenom, antiinflammatory, antitumor properties. Various other effects like immunomodulation, hypolipidemic, antibacterial, cardiovascular protection, sexual behaviour, tolerance and dependence have also been studied. These results are very encouraging and indicate this herb should be studied more extensively to confirm these results and reveal other potential therapeutic effects. Clinical trials using ashwagandha for a variety of conditions should also be conducted.

8. Effect of *Withania somnifera* in pulmonary hypertension [158]

Kashif Hanif et al. Studied the effect of *W. somnifera* root powder on monocrotanile induced PH in rats. This study demonstrated that *W. somnifera* significantly

protected against MCT-induced PH due to its antioxidant, anti-inflammatory, pro-apoptotic and cardio-protective properties.

9. Neuro-protective effects of *Withania somnifera* [159]

Muzamil Ahmad et al. have evaluated the anti-parkinsonian effects of *Withania somnifera* extract, which have been reported to have potent anti-oxidant, anti-proliferative and free radical quenching properties in various diseased conditions. *W. somnifera* extract was found to reverse all the parameters significantly in a dose-dependent manner. This study demonstrated that the extract of *W. somnifera* may be helpful in protecting the neuronal injury in Parkinson's disease.

.10. Protective effect of *Withania somnifera* root powder [160]

M. Rasooi et.al have been evaluate the protective effect of *Withania somsnifera* Dunal (family –Solanaceae), commonly known as Ashwagandha, on adjuvant-induced arthritis rats. Results were compared with those for Indomethacin, a non-steroidal anti-inflammatory drug. Arthritis was induced by intradermal injection of complete Freund's adjuvant into right hind paw of Wister albino rats. The biochemical alterations observed were ameliorated significantly by oral administration of *W. somnifera* root powder (1000mg/kg body weight) in arthritis animals. The results of this study clearly indicate that *W. somnifera* root powder is capable of rectifying the biochemical changes in adjuvant arthritis.

11. *Withania somnifera* on Orofacial – Dyskinesia [161]

Pattipati S. Naidu et.al investigated the role of oxidative stress in the Pathophysiology of haloperidol (HP) - induced orofacial dyskinesia and evaluated the beneficial effect of *Withania somnifera* root extract in the amelioration of HP-induced vacuous chewing movements and tongue protrusions in the rat model for TD. These conclusions strongly suggest that oxidative stress plays a significant role in HP-induced orofacial Dyskinesia and that *W. somnifera* could be effective in preventing neuro-leptic induced extra pyramidal side effects.

12. ***Withania somnifera*** **root extract expounds anti-inflammatory and muco-restorative activity [162]**

Pankaj Pawar et.al investigates the beneficial effects of *W. Somnifera* in Trinitro Benzyl Sulfonic Acid (TNBS) induced experimental IBD through a rectally applicable formulation. Dose of the rectal gel applied at 1000 mf of WSRE per kg rat weight showed significant muco-resttorative efficacy in the IBD-induced rats, validated by histo-pathological studies.

13. Identification of antifungal principle of *Withania somnifera* [163]

Susheel Kumar et al. studied the **e**xtracts of *Chaetomium globosum* EF18, isolated as endophytic fungus from *Withania somnifera*, and were found effective against *Sclerotinia sclerotiorum.* Ethyl acetate and methanol extracts were more effective than hexane extract showing >80% growth inhibition. Bioactive compound (antibiotic Sch 210971, m/z 445 and λ_{max} 290) having antifungal activity against *S. sclerotiorum* has been isolated in pure form from the ethyl acetate extract following bioassay guided fractionation. Ethyl acetate extract was most active having IC_{50} value 35.4 µg/ml.

14. *Withania somnifera* in neuro-behavioral disorder.[164]

Sharanbasappa Durg et.al studied that a systematic search of the effect of *W. somnifera* on brain oxidative stress-induced neuronal pathology was performed using electronic databases. The systematic review was performed on neuro-behavioral parameters; whereas meta-analysis of *W. somnifera* effect was done oxidative stress marker (superoxide dismutase, catalase, and glutathione peroxidase glutathione and lipid peroxidation), nitrite, and protein carbonyl, AchE, ChAT and Ach of rodent brain. Data were analyzed using Review Manager Software.

The systematic review provides scientific evidence for the traditional claim of *W. somnifera* use in different neurological aliments. However, future clinical trials are mandated to establish the therapeutic efficacy and safety in human beings.

15. Anxiolytic-antidepressant activity of *Withania somnifera.*[165]

S.K. Bhattacharya et al. investigated the anxiolytic and antidepressant actions of the bioactive glycol-withanolides, isolated from the roots of *W. somnifera* in rats. The investigation supports the use of *WS* as a mood stabilizer in clinical conditions of anxiety and depression in Ayurveda.

16. *Withania somnifera* to improve Sexual Function [166]

Swati Dongre et al. determined the efficacy and safety of a high-concentration Ashwagandha root extract (HCARE) supplementation for improving sexual function in healthy females. This study demonstrated that oral administration of HCARE may improve the sexual function in healthy women.

17. *Withania somnifera* on tests of cognitive and psychomotor performance[167]

Usharani Pingali et.al: done an assessment of cognitive and psychomotor effects of *Withania somnifera* extract in healthy human participants. These results suggest that *Withania somnifera* extract can improve cognitive and psychomotor performance and therefore be a valuable adjunct in the treatment of diseases associated with cognitive impairment.

18. Examination the effect of *Withania somnifera* supplementation on muscle strength and recovery: a randomized controlled trial [168.]

Wankhade et al. (2015) conducted to examine the possible effect of ashwagandha root extract consumption on muscle mass and strength in healthy men engaged in resistance training program. This study reports that Ashwagandha supplementation is associated with significant increase in muscle mass and strength & suggests that Ashwagandha supplementation may be useful in conjunction with a resistance training program.

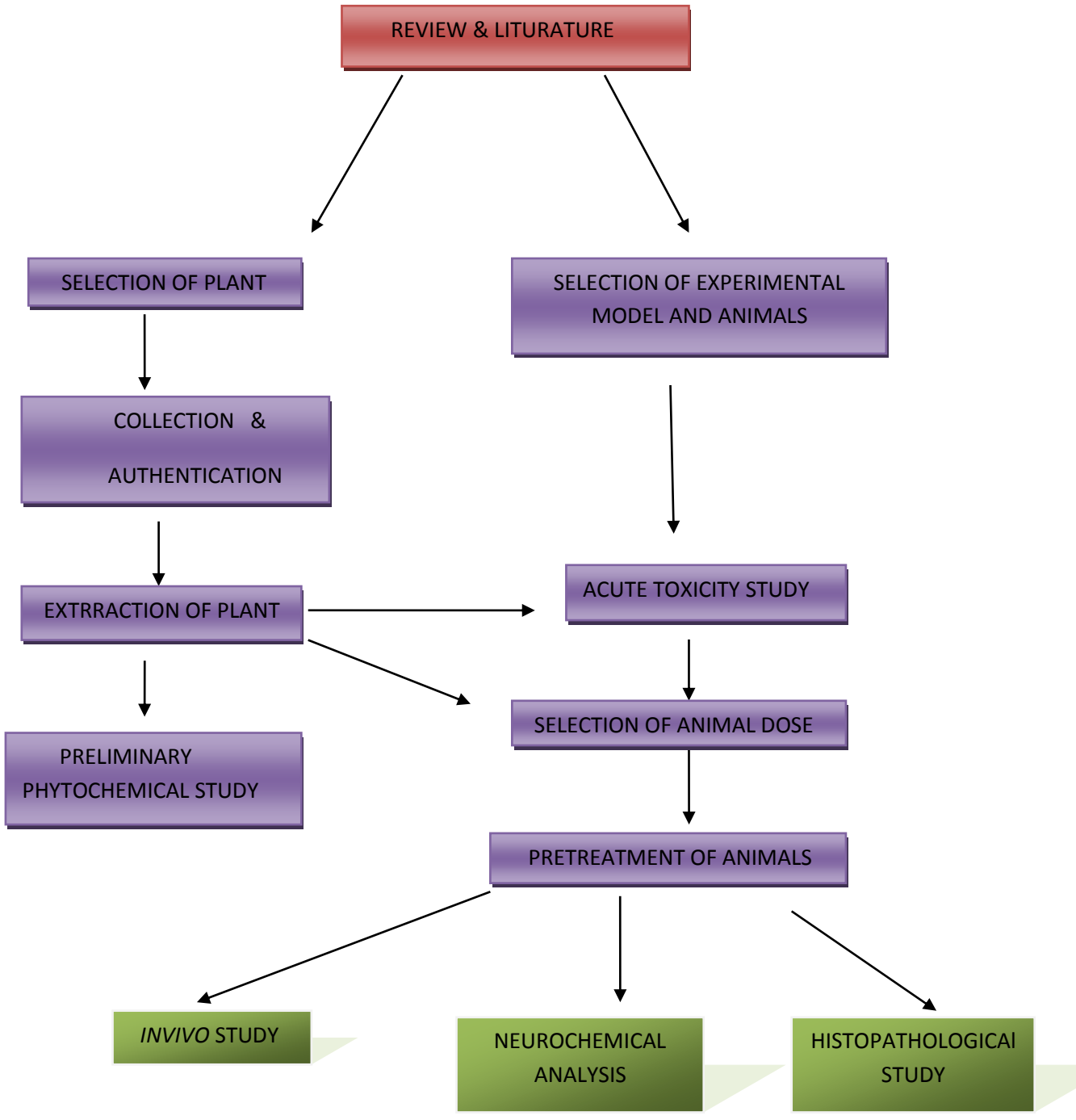
4. SCOPE OF WORK

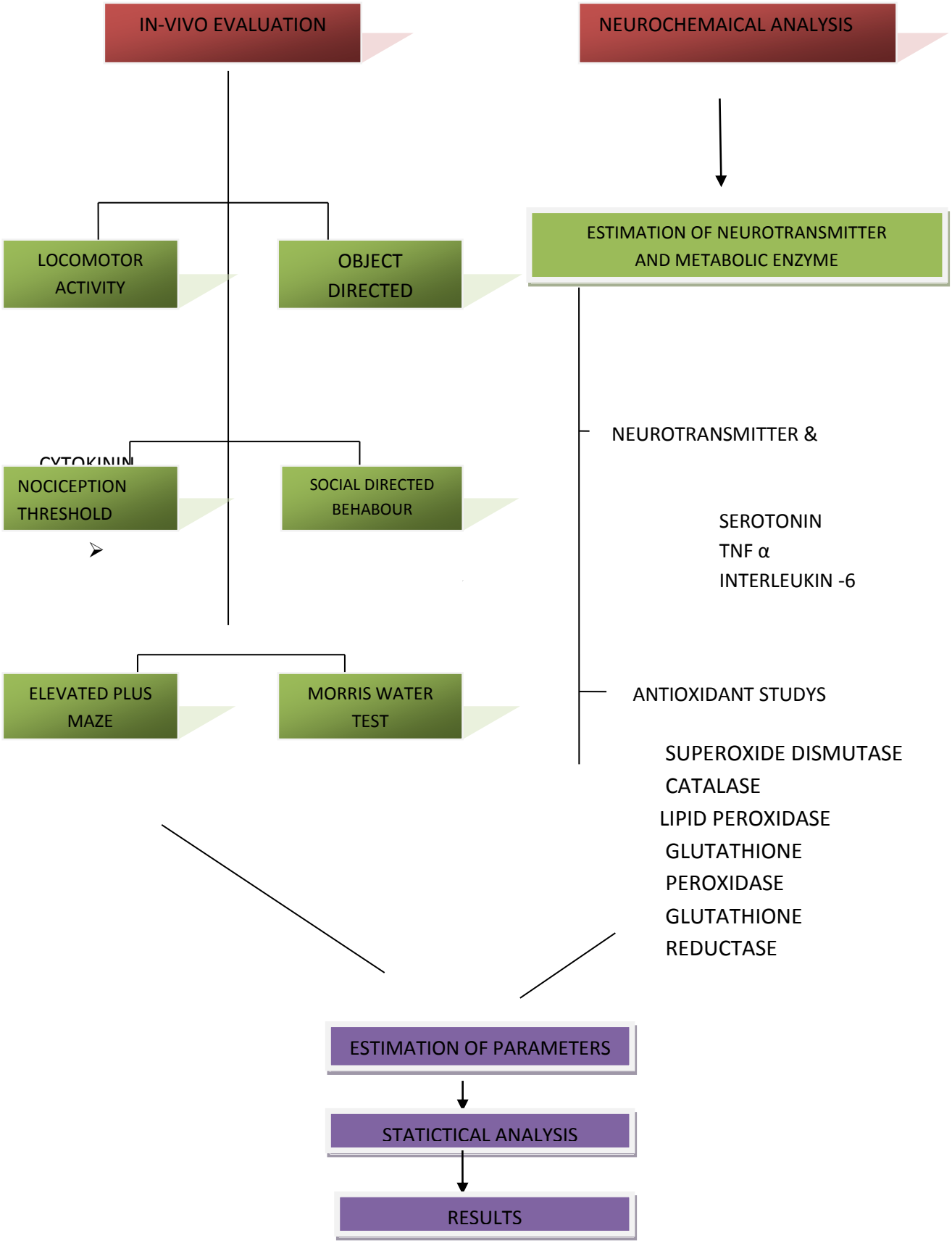
Autism is a neuro-developmental disorder that manifests early in childhood and it is the prevalence in world wide. . The average prevalence of ASD in Asia, Europe and North America was close to 1%. Management of Autism spectrum disorder is a global problem, successful treatment is very important for controlling the raising alarm of the disease.

Till date there is no effective drug available to treat ASD. No herbal drug is proved to manage the severity of symptoms of Autism spectrum disorder. The objective of the present study is, to treat this disorder through nature in the form of herbal medicines or drugs with very minimal adverse effect. ASD among1994-99 born children who turned up adults now were reported with high prevalence. Hence the study was focused on autistic symptoms of adolescent age.

The present study is designed to evaluate the effect of hydro-alcoholic root extract of *Withania somnifera* object-directed behavior, social behavior, cognition development and neuro-inflammation against propionic acid induced Autism Spectrum Disorder (ASD) in mice. So that it may serve clinically for the management of ASD.

5. PLAN OF WORK:




IN-VIVO EVALUATION
NEUROCHEMAICAL ANALYSIS
LOCOMOTOR ACTIVITY
OBJECT DIRECTED
ESTIMATION OF NEUROTRANSMITTER AND METABOLIC ENZYME
NEUROTRANSMITTER &
NOCICEPTION THRESHOLD
SOCIAL DIRECTED BEHABOUR
SEROTONIN
TNF α
INTERLEUKIN -6
ELEVATED PLUS MAZE
MORRIS WATER TEST
ANTIOXIDANT STUDYS
SUPEROXIDE DISMUTASE
CATALASE
LIPID PEROXIDASE
GLUTATHIONE PEROXIDASE
GLUTATHIONE REDUCTASE
ESTIMATION OF PARAMETERS
STATICTICAL ANALYSIS
RESULTS

6. METHODS AND MATERIALS:

6.1. Collection and Authentication

The roots of *Withania somnifera* Dunal (Solanaceae) were collected from local source, Tamil Nadu (Chennai) in March. The plant material was identified and authenticated by Prof P. Jayaraman, PhD, Director: Professor, Presidency College, and Chennai 600005. PLANT ANATOMY RESEARCH CENTRE. Tambaram, Chennai-5, Tamilnadu. [NO: NISMB1592015]. A voucher specimen was submitted at C.L. Baid Metha College of Pharmacy, Chennai-97.

6.2 Preparation of Hydro Alcoholic Root Extract of *Withania somnifera*

The dried roots of *Withania somnifera* were collected. The dried root was made into coarse powder. The powdered roots of about 200g were extracted with hydro alcohol (ethanol/water – 80:20) in maceration process. After extraction the solvent was evaporated at 40 ºC to obtain a viscous mass.

6.3 CHEMICALS:

All the Chemicals used in the study were of analytical grade. The following chemicals were used for the experimental study.

Table 1: Name of the chemicals and their source

Sl .No	MATERIALS	SOURCE
1	Acetic acid	S.d.fine chemicals Ltd, Mumbai
2	Chloroform	S.d.fine chemicals Ltd, Mumbai
3	Disodium EDTA	Chemspure, Chennai
4	Distilled water	Andavar distilled water
5	Ethanol	Cisco research lab.Mumbai
6	$Fecl_3$ reagent	S.d.fine chemicals Ltd, Mumbai
7	Formaline	S.d.fine chemicals Ltd., Mumbai
8	Glacial acetic acid	Chemspure, Chennai
9	Glutathione oxidase	Qualigens fine chemicals, Mumbai
10	Glutathione reductase	Qualigens fine chemicals,Mumbai
11	Heptanes	Loba chemicals Ltd, Mumbai
12	Hydrochloric acid	Loba chemicals Ltd, Mumbai
13	Hydrogen peroxide	Qualigens fine chemicals, Mumbai
14	Iodine	Chemspure, Chennai
15	n-butanol	S.d.fine chemicals Ltd, Mumbai
16	Nicotinamide adenine dinucleotide phosphate reduced tetra sodium($NADPH.Na_4$)	S.d.fine chemicals Ltd, Mumbai
17	Perchloric acid	Loba chemicals Ltd, Mumbai
18	Potassium chloride	Chemspure, Chennai
19	Potassium dihydrogen phosphate	S.d.fine chemicals Ltd, Mumbai

20	Propionic acid	Merck Specialties private Ltd,Mumbai
21	Pyridine	Fischer inorganic& aromatics Ltd, Chennai
22	Sodium acetate	S.d.fine chemicals Ltd, Mumbai
23	Sodium azide	S.d.fine chemicals Ltd, Mumbai
24	Sodium chloride	Paxy specialities Pvt Ltd
25	Sodium dodecyl sulphate (SDS)	S.d.fine chemicals Ltd, Mumbai
26	Sodium hydrogen carbonate	S.d.fine chemicals Ltd, Mumbai
27	Sodium hydroxide pellets	Chemspure, Chennai
28	Sodium thiosulphate	S.d.fine chemicals Ltd, Mumbai
29	Sulphuric acid	S.d.fine chemicals Ltd, Mumbai
30	Thiobarbituric acid	Rolex laboratory reagent, Mumbai
31	Thionyl chloride	S.d.fine chemicals Ltd, Mumbai
32	Trichloro acetic acid	Span diagnosis Ltd, Bangalore

6.4. PRELIMINARY PHYTOCHEMICAL ANALYSIS [169]

The Hydroalcoholic extract of *Withania somnifera* (HAREWS) was subjected to preliminary phytochemical screening for the presence or absence of phyto-constituents by the following methods.

1.Test for alkaloids:

The extract was treated with dilute hydrochloric acid and filtered. The filtrate is used in the following tests.

a) Mayer's reagent (Potassium Mercuric Iodine Solution)

0.5ml of the extract was treated with Mayer's reagent and the appearance of cream color indicates the presence of alkaloid

b) Dragendroff's test (Potassium Bismuth Iodide)

0.5ml of the extract was treated with Dragen droff's reagent and the appearance of reddish brown color precipitate indicates the presence of alkaloid.

c) Hager's test (Saturated solution of Picric acid)

0.5ml of the extract was treated with Hager's test and the appearance of yellow color precipitate indicates the presence of alkaloid.

d) Wagner's test (Iodine-Potassium Iodide Solution)

0.5ml of the extract was treated with Wagner's test and the appearance of brown color precipitate indicates the presence of alkaloid.

2) Test for Carbohydrates

a) Molisch's test:

The extract was treated with 3ml of alpha-napthol in alcohol and concentrated sulphuric acid was added along the sides of the test tube carefully. Formation of violet color ring at the junction of two liquids indicates the presence of carbohydrates.

b) Fehling's test (CuSO4.7H2O+KOH+Potassium Tartarate):

The extract was treated with Fehling's solution A and B heated in boiling water for few minutes. The appearance of reddish brown color precipitate indicates the presence of reducing sugars.

c) Benedict's test (Sodium citrate + sodium carbonate + CuSO4.7H2O)

The extract was treated with Benedict's test and heated in boiling water for few minutes. The appearance of reddish orange color precipitate indicates the presence of reducing sugars.

d) Barfoed's test (Copper Acetate+ Glacial acetic acid)

The extract was treated with Barfoed's test and heated in boiling water for few minutes. The appearance of reddish orange color precipitate indicates the presence of non-reducing sugars.

3) Test for steroids

a) Libermann Burchard test:

The extract was treated with small quantity of concentrated sulphuric acid, glacial acetic acid and acetic anhydride. The appearance of green color indicates the presence of steroids

4) Test for proteins

a) Biuret's test:

The extract was treated with copper sulphate and sodium hydroxide solution. The appearance of violet color indicates the presence of proteins.

b) Millon's test:

The extract was treated with Millon's reagent. The appearance of pink color indicates the presence of proteins.

5) Test for Tannin's

a) The extract was treated with 10% lead acetate solution. The appearance of white precipitate indicates the presence of tannins.

b) The extract was treated with aqueous bromine solution. The appearance of white precipitate indicates the presence of tannins.

6) Test for Phenols

a) The extract was treated with neutral ferric chloride solution. The appearance of violet indicates the presence of phenols.

b) The extract was treated with 10% sodium chloride solution. The appearance of cream color indicates the presence of phenols.

7) Test for Flavonoid's

a) 5ml of extract solution was hydrolysed with 10%v/v sulphuric acid and cooled. Then, it is extracted with diethyl ether and divided into three portions in three separate test tubes. 1ml of diluted sodium carbonate, 1ml of 0.1N sodium hydroxide, and 1ml of strong ammonia solution were added to the first, second and third test tubes respectively. In each test tube, development of yellow color demonstrated the presence of flavonoids.

b) Shinoda's test: The extracts were dissolved in alcohol, to that one piece of magnesium is added followed by concentrated hydrochloric acid along the sides of the test tube drop wise. It is heated in a boiling water bath for few minutes. The appearance of magenta colour indicates the presence of flavonoids.

8) Test for Gums and Mucilage

The extract was treated with 25ml of absolute alcohol and then solution was filtered. The filtrate was examined for its swelling properties.

9) Test for Glycosides

The extract was dissolved in the glacial acetic acid and few drops of ferric chloride solution was added, followed by the addition of concentrated sulphuric acid, formation of red ring at the junction of two liquids indicates the presence of glycosides.

10) Test for Saponins

1ml of the extract was diluted to 20ml with distilled water and shaken well in a test tube. The formation of foam in the upper part of the test tube indicates the presence of saponins.

11) Test for Terpenes

The extract was treated with tin and thionyl chloride, appearance of pink color indicates the presence of terpenes.

12) Test for sterols

The extract was treated with 5% potassium hydroxide solution; appearance of pink color indicates the presence of sterols.

6.5. ACUTE TOXICITY STUDIES [170]

The procedure was followed by using OECD 423 guidelines 423.The acute toxic class method (423) is a step wise procedure with 3 animals of single sex per step. Depending on the mortality and/or morbidity status of the animals, on the average 2-4 steps may be necessary to allow judgment on the acute toxicity of the substances. This procedure results in the use of a specified number of animals while allowing for acceptable data- based scientific conclusion.

The method used defined doses (2000mg/kg body weight) and results allow a substance to be ranked and classified according to the Globally Harmonized System (GHS) for classification of chemical which cause acute toxicity.

Procedure:

Adult female Swiss albino mice 25- 35gms were used for the study. The starting dose level of Hydro-alcoholic root extract of *Withania somnifera* (HAREWS) was 2000mg/kg body weight p.o. as most of the crude extracts possess LD50 value more than 2000 mg/kg, p.o. so starting dose used was 2000mg/g p.o. Dose volume administered was 1ml/100 gm body weight to mice which were fasted overnight with water *ad libitum*. Food was withheld for further 3-4hrs after oral administration of drugs and observed for the signs of toxicity.

Body weight of mice's before and after determination were noted and any changes in skin and fur, eyes and mucous membrane, respiratory, circulatory, autonomic & central nervous system, motor activity and behavior pattern were observed and also sign of tremors, convulsion, salivation, diarrhoea, lethargy, sleep and coma were noted. The onset of toxicity and signs of toxicity also noted.

6.6. EXPERIMENTAL DESIGN

Swiss albino male mice were obtained at age 26 days from the animal house of C.L.Baid Metha College of Pharmacy, Thoraipakkam, Chennai- 97, and housed in groups of six at 22±2◦C in acrylic cages (26cm×48cm×21 cm) for 7 days for acclimation to the animal colony, with 12hrs light/dark cycle and access to pellet chow and water ad libitum. Post-surgical housing was individual for 7 days to allow recovery.Ethical committee clearance was obtained from IAEC (Institutional Animal Ethics Committee) of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

IAEC Reference: IAEC/XLVII03/CLBMC P/2015

After recovery of mice (41±4days old), the animals were assigned as follows:

GROUP	TREATMENT	DURATION
Group I – Normal control	Normal saline, p.o	For 28 days
Group II – Negative control	Propionic acid (1µl of 0.26M solution), icv.	For 7 days (2dose/day); From 22nd to 28th day
Group III – Low dose	HAREWS (200mg/kg), p.o	For 28 days
Group IV – High dose	HAREWS (400mg/kg), p.o	For 28 days

6.7. INDUCTION OF ASD

Cannula implantation [171, 172]

For surgery a mouse was anaesthetized with 4% isoflurane and 2 L/min oxygen and placed in a standard stereotaxic device, with maintenance of anaesthesia. The cannula, a 30-gauge needle, at angle at 9° was implanted 3mm deep, into the left ventricle with the following co-ordinates with reference to bregma: midline, anterior/posterior -0.3mm, medial/lateral 1.3mm. Four small stainless steel screws were inserted into the skull surrounding the cannula to provide anchors for dental acrylic, which attached the cannula to the skull. The gauge injection cannula was connected to a Sage syringe pump by PE10 tubing. A removable plug sealed the guide cannula until an injection was to be made. Immediately after post-surgery, all mice received a subcutaneous injection of analgesic (pentazocine, 10mg/kg).

ICV infusion of propionic acid (PPA) [171]

All groups except normal control received intracerebro ventricular (icv) infusions twice daily, separated by 4 h, for seven consecutive days. Behavioural test sessions were carried out immediately after icv infusion of 1µl of 0.26M propionic acid (Solutions were buffered to physiological pH 7.5 before injection using hydrochloric acid or sodium hydroxide.). Each injection consisted of 1µl of solution delivered over a period of 1min. To ensure that the entire injection had been delivered, the injection cannula was allowed to remain in place for an additional minute before being removed. The behavioural test sessions were separated by a 1 week recovery period

All mice were used in all behavioural tests, with PPA administered shortly before each test except to the normal control group. Test sessions were separated by intervals of 24 or 48 h, as indicated below, because of an active period of PPA in brain for producing behavioural and brain electrographic changes lasting no more than 40–60 min.

6.8. ASSESSMENT OF HABITUATION BEHAVIOUR

1.Locomotor, repetitive/stereotypic behavior [173]

The locomotor activity of mice is recorded individually for each animal in an actophotometer. It was recorded on 22nd and 26th day of experiment. It is equipped with 3 infrared emitters located on the x- and y- axis, and with an equivalent number of receivers on the opposite walls of the cage. The behavior of mice was analyzed. The locomotor activity was defined as a breakage of three consecutive photo- beams. The time of repetitive/stereotypic- like activity was defined as the sum of time intervals (1/10th of a second) in which there was a movement, but an animal did not cross three consecutive photo-beams. The animal would have to repeatedly break and make the same three beams for the time interval (1/10th of a second) to be recorded as time of repetitive/stereotypic-like activity. The number of repetitive/stereotypic-like movements was defined as the number of repeated breaks of the same beam in 1/10th of a second.

2.Nociception threshold [173.174]

Nociceptive effects were evaluated on 22nd and 27th day using tail flick. The tail flick test is to be carried out by restraining the animal by hand, and its tail was immersed ≈2 cm into water heated to 50±5°C. After tail immersion, the time at which tail flick response was noted was recorded. The cutoff time is 15s. Tail flick measurements are taken three times at 30s intervals to obtain average pain threshold of mice.

3. Object-directed behavior[13]

The novel objects, three different small toys were placed equidistant from each other approximately 10cm from the wall of the open-field arena. After administration of the assigned treatment on 23rd and 25th day, the mouse was placed at the center of the arena and allowed to explore and interact with the objects for 5min. Each mouse should be tested once and the same 3 objects are used for all tests, counterbalanced for position within groups. A 20cm-diameter zone around each object is defined and the percentage approach into, and total duration(s) in each zone is determined. Sniffing bouts at each object by each mouse is recorded. A sniffing bout was scored when a mouse approached an object with its snout

placed within 1cm of the object and with the vibrissae moving to indicate sniffing, and ended when the snout is withdrawn farther than 1cm from the object.

4. Novel mice vs. novel object-directed behavior [136]

This test is carried out 24h or 48hrs after the object-directed behavior test and evaluated social for behavior. The test was carried out on 24th and 27th day. A novel male mouse of the same body weight as the subject mouse, and an object not used in the previous object-directed behavior test, are placed opposite each other in the arena approximately 10cm from the wall of the arena. The novel mouse movements are restricted by placing it in the small wire mesh cage.

The subject mouse is placed at the center of the arena midway between the novel object and the novel mouse facing the bare wall of the arena, and allowed to explore for 5min. Mice are tested one at a time, and the arena and object were cleaned with an alcohol–water solution after each mouse is tested. Each mouse is tested once and the same object was used for all tests, with stimulus object/mice positions counterbalanced within groups. Percent of time approaching the novel mouse 1) the novel object 2) by the subject mouse is calculated.

5. Elevated Plus Maze [175]

On 28th day, all mice were subjected to elevate plus maze for 5min to assess anxiety level. Maze is composed of 2 open arms (16x5cm) and 2 closed arms (16x5x12cm) with open roof top. The maze is elevated at the height of 25cm. Each animal was placed at the center of the maze facing towards open arm. The following parameters are recorded: (a) transfer latency to enter open arm, (b) time spent in open arm, (c) number of entries into open arm in 5min of the session.

6.9. ASSESSMENT OF MEMORY AND RETENTION

Morris water maze [176,177]

The Morris water maze method is performed to evaluate spatial working and reference memory. The experimental apparatus consists of a circular tank (120 cm in diameter, 45 cm in height). An invisible platform (15 cm in diameter, 35 cm height) was placed 1.5 cm below the surface of the water. Water was kept opaque by dissolving small quantity of milk at a temperature of 21-23ºc. The pool was located in a test room and many cues external to the maze were visible from the pool, which could be used by the mice for spatial orientation. The position of the cues was kept constant through the task. The training trials were carried out from 26th to 28th day- 4 trials per day. The platform is located in a constant position throughout the test period in the middle of one quadrant, equidistant from the center and edge of the pool. In each training session, the latency to escape to the hidden platform was recorded.

On 28th day, the platform was removed and the animals were tested for its memory where the time spent by each animal in target quadrant searching for the hidden platform is noted as an index of retrieval.

6.10. ESTIMATION OF BRAIN NEUROTRANSMITTER

Estimation of serotonin [178]

Reagents:

1. HCl- Butanol sol: (0.85ml of 37% hydrochloric acid in one-liter *n*-butanol)

2.Heptanes

3.0.1 M HCl : (0.85ml of conc. HCl upto 100ml H_2O)

4. O-phthaldialdehyde (OPT) reagent (20mg in 100ml conc. HCl)

Procedure

On the 28th day of experiment, immediately after MWM, mice were sacrificed, whole brain was dissected out and the sub cortical region (including the striatum) was separated. Weight tissue was weight and was homogenized in 5ml HCl- butanol for about 1 min. The sample was then centrifuged for 10 min at 2000 rpm. An aliquot supernatant phase (1 ml) was removed and added to centrifuge tube containing 2.5 ml heptanes and 0.31 ml HCl of 0.1M. After 1min of vigorous shaking, the tube was centrifuged under the same conditions above in order to separate the phases, and the overlaying organic phase was discarded. The aqueous phase (0.2ml) was then taken for 5HT assay. All steps were carried out at 0^0C. (N.B: it taken in between 50-75mg of tissue for homogenate with 5ml of HCl-butanol in correlation of same tissue concentration 1.5-5mg/0.1ml of HCl-butanol used in Schlumpf M *et al*, 1974. This is done to get adequate amount of supernatant liquid for analysis)

To 0.2ml aqueous extract, 0.25ml of OPT reagent was added. The fluorophore was developed by heating to 100°C for 10min. After the samples reached equilibrium with the ambient temperature, readings were taken at 360-470nm in the spectrofluorimeter. Tissue blank, 0.25ml conc. HCl without OPT was added. Internal standard: 500µg/ml was prepared in distilled water: HCl-butanol in 1:2 ratio.

6.11. Estimation of Neuroinflammation

Estimation of cytokine: TNFα [179]

Reagents

1.50mM Tris (pH 7.2)

2.1mM EDTA

3.6mM $MgCl_2$

4.5% protease inhibitor cocktail

Procedure

Brain tissue was homogenized in 1ml ice cold buffer (pH 7.2, 4^oC) having 50 mM Tris, 1mM EDTA, 6mM $MgCl_2$, 5%(w/v) protease inhibitor cocktail. Then samples were sonicated and centrifuged at 20, 800g for 20 min in cooling centrifuge. The supernatants were used for the determination of TNF-α using commercially available ELISA kit obtained from Ray Biotech, USA. The procedures were followed as per the manufacturer's instructions. All data are expressed as pg/ml.

Estimation of cytokine: Interleukin 6 (IL-6) [180]

Reagents

1. RIPA buffer (50mM Tris-Hcl,150mM Nacl pH-7.4)

2.1mM EDTA

3.1mM PMSF

4.Protease inhibitor cocktail.

Procedure

One half of the brain tissue was homogenized using disposable rotor homogenizer in 10 volumes of RIPA (radio-immune precipitation assay) buffer (50mM Tris –Hcl, 150mM Nacl, pH 7.4) supplemented with 1mM EDTA, 1mM PMSF(phenyl methyl sulpfonyl fluoride) and protease cocktail was separated to sub cellular fraction..The homogenate centrifused at 29000g for 20min. The pellet was resuspended with RIPA buffer containing 1mM EDTA and protease cocktail, whilst the supernatant was centrifuged at 29000 for 45min.the supernatants was used for the determination of IL-6 by specific quantitative sandwich ELISA kits according to the manufacturer's instruction.

6.12. ESTIMATION OF ANTIOXIDANT ENZYMES

Estimation of Superoxide dismutase (SOD) [181]

Reagents

1. Carbonate buffer (100mM, pH 10.2)
2. Epinephrine (3mM)

Procedure

The SOD activity in supernatant was measured by the method of Misra and Fridovich. The supernatant (500µl) was added to 0.800ml of carbonate buffer (100mM, pH 10.2) and 100µl of epinephrine (3mM). The change in absorbance of each sample was then recorded at 480nm in spectrophotometer for 2min at an interval of 15sec. Parallel blank and standard were run for determination of SOD activity.

One unit of SOD is defined as the amount of enzyme required to produce 50% inhibition of epinephrine auto oxidation.

Reagents	Uninhibited (Standard)	Inhibited (Sample)	Blank
Carbonate buffer	0.900ml	0.800ml	1.0ml
Supernatant	-	0.1ml	-
Epinephrine	0.1ml	0.1ml	-

The reaction mixtures are diluted 1/10 just before taking the readings in spectrophotometer

Calculation

$$\%\mathbf{Inhibition} = \frac{\Delta A_{480nm} \quad /\min \text{ Uninhibited} - \Delta A_{480nm}/\min \text{ inhibited}}{\Delta A_{480nm} \quad /\min \text{ Uninhibited} - \Delta A_{480nm}/\min \text{ Blank}} \times 100$$

$$\mathbf{Units} \quad /\mathbf{ml\ enzyme} = \frac{\%\text{Inhibition} \times V_t}{(50\%) \times V_s}$$

$$\mathbf{Units} \quad / \quad \mathbf{mgprotein} = \frac{\text{Units} \quad / \text{ ml enzyme}}{\text{mg protein} \quad / \quad \text{ml enzyme}}$$

Where,

V_t = Total volume (1.0ml)

V_s = Sample volume (0.1ml)

Estimation of Catalase (CAT) [182]

Reagents

1.Phosphate buffer solution (50mM)

i. Dissolve 6.81g of KH_2PO_4 in 1000ml distilled water
ii. Dissolve 6.9g of Na_2HPO_4 in 1000ml distilled water

390ml from solution (A) are mixed with 610ml from solution (B), the pH is adjusted to 7

2.Hydrogen peroxide (H_2O_2) 30mM

0.34ml of 30% H_2O_2 is diluted with phosphate buffer to 100ml

Procedure

Catalase activity was measured by the method of Aebi. 0.1ml of supernatant was added to cuvette containing 1.9ml of 50mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0ml of freshly prepared 30mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240nm. Activity of catalase was expressed as units/mg protein. A unit is defined as the velocity constant per second.

Reagents	Sample	Blank
Phosphate buffer	1.9ml	2.9ml
Supernatant	0.1ml	0.1ml
H_2O_2	1ml	-

The reaction occurs immediately after the addition of H_2O_2.

Solutions are mixed well and the first absorbance (A_1) is read after 15sec (t_1) and the second absorbance (A_1) after 30sec (t_1). The absorbance is read at wavelength 240nm.

Calculation

$$\mathbf{K} = \frac{V_t}{V_s} \times \frac{2.3}{\Delta t} \times \log \frac{A_1}{A_2} \times 60$$

Where,

K =Rate constant of the reaction

$\Delta t = (t_2 - t_1) = 15$sec

A_1 = Absorbance after 15sec

A_2 = Absorbance after 30sec

V_t = Total volume (3ml)

V_s = Volume of the sample (0.1ml)

Estimation of Lipid peroxidase (LPO) [183]

The level of Lipid peroxidase was estimated by Thiobarbituric acid reaction method described by Ohkawa *et al.*

Reagents

1. Sodium dodecyl sulphate (SDS) (8.1% w/v)
2. Acetic acid (20%; pH 3.5)
3. Thiobarbituric acid (TBA) (0.8%)
4. n-butanol/pyridine mixture (15:1)

Procedure

To 0.2ml of test sample, 0.2ml of SDS, 1.5ml of acetic acid and 1.5ml of TBA were added. The mixture was made up to 4ml with water and then heated in a water bath at 95°C for 60min. After cooling, 1ml of water and 5ml of n-butanol/pyridine mixture were added and shaken vigorously. After centrifugation at 4000rpm for 10min, the organic layer was taken and its absorbance was read at 532nm. The level of lipid peroxides was expressed as nmoles of MDA released/g wet tissue.

Reagents	Sample	Blank
SDS	0.2ml	0.2ml
Supernatant	0.2ml	-
DDW	1.6ml	1.8ml
Acetic acid	1.5ml	1.5ml
TBA	1.5ml	1.5ml
n-butanol/pyridine	5ml	5ml

Calculation

$$\textbf{Concentration of MDA} = \frac{\text{Absorbance at 532nm}}{\text{L} \times \epsilon} \times \text{D}$$

Where,

L = Light path (1cm)

ϵ = Extinction co-efficient 1.56×10^5 $M^{-1}cm^{-1}$

$$\text{D} = \frac{\text{Total volume (10ml)}}{\text{Volume of the sample (0.2ml)}}$$

Estimation of Glutathione Peroxidase [184]

Reagents

1. Phosphate buffer, pH 7.0 (75mM)
2. Glutathione reductase (60mM)
3. Sodium azide (0.12M)
4. Disodium EDTA (0.15mM)
5. NADPH (3mM)
6. H_2O_2 (7.5mM)

Procedure

3ml cuvette containing 2.0ml of phosphate buffer (75mmol/L, pH 7.0), 50µl of glutathione reductase (60mmol/L), 50µl of NaN_3 (0.12mol/L), 0.1ml of Na_2EDTA (0.15mM/L), 100µl of NADPH (3.0mmol/L) and tissue supernatant were added. Water was added to make a total volume of 2.9ml. The reaction was started by the addition of 100µl of (7.5mmol/L) H_2O_2, and the conversion of NADPH to NADP was monitored by a continuous recording of the change of absorbance at 340nm at 1min interval for 5min. Enzyme activity of GSHPx was expressed in terms of mg of protein.

Reagents	Sample	Blank
Phosphate buffer	2.0ml	2.9ml
Glutathione reductase	0.05ml	0.05ml
Sodium azide	0.05ml	0.05ml
Disodium EDTA	0.1ml	0.1ml
NADPH	0.1ml	0.1ml
Supernatant	0.1ml	0.1ml
H_2O_2	0.1ml	-

DDW	0.5ml	0.6ml

Calculation

$$\textbf{Enzyme activity}(\textbf{M/min/ ml}) = \frac{A_{340} / \min \times V_t}{\varepsilon \times d \times V_s}$$

Where,

$\varepsilon = 6.22 \times 10^6\ M^{-1}cm^{-1}$

d = 1cm

V_t = Total volume (3.0ml)

V_s = Sample volume (0.1ml)

Estimation of Glutathione Reductase (GRD) [185]

Glutathione reductase was assayed by the method of Stahl *et al*.

Reagents

1. Phosphate buffer (0.3M; pH 6.5)
2. EDTA (0.25M)
3. Glutathione oxidized, GSSG (0.012M)
4. NADPH (0.03M); Nicotinamide Adenine Dinucleotide Phosphate reduced tetra sodium salt, NADPH.Na_4 (Mw.833.35)

Procsedure

The reaction mixture containing 1ml phosphate buffer, 0.5ml EDTA, 0.5ml GSSG and 0.2ml of NADPH was made up to 3ml with distilled water. After the addition of 0.1ml of tissue homogenate, the change in optical density at 340nm was monitored for 2min at 30sec interval.

One unit of the enzyme activity was expressed as nmoles of NADPH oxidized/min/mg protein.

Reagents	Sample	Blank
Phosphate buffer	1.0ml	1.5ml
EDTA	0.5ml	0.5ml
GSSG	0.5ml	-
NADPH	0.2ml	0.2ml
Supernatant	0.1ml	0.1ml
DDW	0.8ml	0.8ml

Calculation

$$\textbf{Enzyme activity}(\textbf{M/min/ ml}) = \frac{A_{340} / \min \times V_t}{\varepsilon \times d \times V_s}$$

Where,

$\varepsilon = 6.22 \times 10^6\ M^{-1}cm^{-1}$

d = 1cm

V_t = Total volume (3.1ml)

V_s = Sample volume (0.1ml)

6.13. Methods for Histopathological study

The mice from each group were anaesthetized using intraperitoneal injection of sodium pentobarbital (50mg/kg). The brain was carefully removed without injury after opening the skull. The collected brain was washed with ice cold normal saline and fixed in 10% formal saline (10ml of formaldehyde in 90ml of physiological saline). Paraffin embedded sections were taken 100μm thickness and processed in alcohol-xyline series and stained with haematoxylin-eosin dye. The sections were examined microscopically for histopathological changes.

6.14. Statistical Analysis

The statistical analysis was carried by one way ANOVA followed by Dunnet's "t" test. P value <0.05 (95% confidence limit) was considered statistically significant, using software Graph Pad Prism version6.

RESULTS:

7.1 Extraction yield

The hydro-alcoholic root of *Withania somnifera* dried extract was dark chocolate brown in colors and was stored in refrigerator until use. The percentage yield of the extract was 15.02% w/w.

7.2 Preliminary Phytochemical study:

The hydro-alcoholic root extract of *Withania somnifera* showed presence of various Phyto-chemical constituents such as alkaloids, carbohydrate, proteins, tannins, steroids, glycosides, flavonoids, and saponins. Results are shown in **Table 1**

7.3. Acute Toxicity study:

According to the OECD guideline 423 (Acute toxic class method) acute oral toxicity studies were performed. A single starting dose of 2000mg/kg p.o was administered to the animal and observed for three days. There was no significant change in body weight of the animals before and after treatment of drug and no signs of toxicity was found and the animals were observed for 14 days. No signs of toxicity were found. Results are shown in **Table 2**.

7.4. Assessment of Habituation behavior:

Effect of HAREWS on Locomotor, repetitive/stereotypic behavior:

The Group II animals showed a significant decrease in locomotor activity compared to the Group I animals. Treatment with HAREWS (200&400 mg/kg) significantly increased the locomotor activity (Group III & Group IV) on comparison with Group II. There was significant difference in the repetitive / stereotypic behavior of both Group I and Group II animals. Treatment with HAREWS (200& 400mg/kg) significantly decreased the number and duration of repetitive / stereotypic activity. Results are given in **Table4** and represent **Graph2**

Effect of HAREWS on Nociception thresholds:

The Group II animal shows increase in nociception threshold when compared with the Group I. Treatment with HAREWS (200&400mg/kg) significantly decreased the nociception threshold when compared with the Group II. Results are shown in **Table5** and represent **Graph 3**

Effect of HAREWS on object directed behavior

There was a significant increase in object directed activity by Group II when compared with Group I and significant decrease when compared with Group III and IV. Treatment with HAREWS (200mg/kg &400mg/kg) object directed behavior significantly decreased. Percentage object directed behavior, number of sniffing bouts and their spent time in each zone is given in **Table 6, 7, 8** respectively and **Graph 4, 5, 6.**

Effect of HAREWS on social directed behavior

There was a significant decrease in percentage approach towards novel mice by Group II when compared with Group I. Treatment with HAREWS (200mg/kg &400mg/kg) showed significant increase in percentage approach to novel mice on comparison with Group II. Results are given in **Table 9** and represent in **Graph 7**.

Effect of HAREWS on elevated plus maze

There was no significant difference between Group II & Group I, Group III & Group IV in number of entries, time spent and transfer latency to open arm. But Group I showed significant difference on time spent in open arm when compared with Group II. Results are given in **Table 10** and represented in **Graph 8, 9.**

7.5 Assessment of memory and retention

Effect of HAREWS of Morris water maze test:

The Group II animal showed increase in latency period on comparison with Group I animals. Treatment with HAREWS 200mg/kg & 400mg /kg decreased the latency time significantly (Group III and Group IV) on comparison with the Group II. Results are given in **Table 11** and represented in **Graph 10**

7.6. Estimation of Neurotransmitter

Effect of HAREWS on Serotonin

The serotonin level in the brain of Group II animals was increased significantly on comparison with Group I animals. All treatment groups treated with HAREWS 200mg/kg & 400mg/kg (Group III & Group IV respectively) exhibited significant decrease in the level of serotonin in comparison with Group II animals. Results are given in **Table 12** & represented in **Graph11**.

7.7. Estimation of Neuroinflammation:

Effect of HAREWS on TNF-α

The brain TNF-α level of Group II animals were increased significantly on comparison with Group I animals. All treatment groups treated with HAREWS200mg/kg & 400mg/kg (Group III & Group IV respectively) exhibited significant decrease in the level of TNF-α in comparison with Group II animals. Results are given in **Table 13** & represented in **Graph 12.**

Effect of HAREWS on Interleukin 6 (IL-6)

The IL-6 level in the brain of Group II animals was significantly increased on comparison with the Group I animals. All treatment Groups treated with HAREWS 200mg/kg & 400mg/kg (Group III& Group IV) showed significant decrease in the level of IL-6 in comparison with Group II animals. Results are given in **Table14** & represented in **Graph 13.**

7.8. Estimation of Antioxidant Enzymes

Effect of HAREWS on Superoxide dismutase

SOD level in the brain of Group II animals was decreased significantly on comparison with Group I animals. Treatment with HAREWS 200mg/kg and 400mg/kg (group III & Group IV) showed significant increase in SOD level on comparison with Group II animals. Results are given in **Table15** and **Graph14.**

Effect of HAREWS on Catalase

Catalase level in the brain of Group II animals were found decreased significantly on comparison with Group I animals. Treatment with HAREWS 200 and 400mg/kg (Group III & Group IV) showed significant increase in catalase level on comparison with Group II animals. Results are given in **Table16** and represented in **Graph 15.**

Effect of HAREWS on Lipid peroxidation

Lipid peroxidation in the brain of Group II animal were found increased significantly on comparison with HAREWS 200& 400mg/kg (Group III & Group IV) showed significant decrease in lipid peroxidation on comparison with Group II animals. Results are given in **Table17** and represented in **Graph 16**

Effect of HAREWS on Glutathione peroxidase

Glutathione peroxidase in the brain of Group II animals were found decreased significantly on comparison with Group I animals. Treatment with HAREWS 200 mg/kg & 400mg/kg (Group III & Group IV) showed significant increase in Glutathione peroxidase level on comparison with Group II animals. Results are given in **Table18** & represented in **Graph 17**

Effect of HAREWS on Glutathione reductase

Glutathione reductase in the brain of Group II animals were found decreased significantly on comparison with Group I animals. Treatment with HAREWS 200 & 400mg/kg (Group III & Group IV) showed significant increase in Glutathione reductase level on comparison with Group II animals. Results are given in **Table 19** & represented in **Graph 18**

7.8. Tables & Graphs:

Table 2: Preliminary Phytochemical study

Sl.No	Constituents	Remarks
1	Alkaloids	Present
2	Carbohydrates	Present
3	Proteins	Present
4	Steroids	Present
5	Phenols	Absent
6	Tannins	Present
7	Glycosides	Present
8	Flavonoids	Present
9	Saponins	Present
10	Terpens	Absent
11	Gum & Mucilage	Absent
12	Sterols	Absent

Table 3: Acute oral toxicity results of HAREWS

Sl No	Treatment	Dose	Weight of animals		Sign of toxicity	Onset of toxicity	Reversible or Irreversible	Duration
			Before	After				
1	HAREWS	2000mg/kg	30	32	No sign of toxicity	Nill	Nill	14 days
2	HAREWS	2000mg/kg	25	28				
3	HAREWS	2000mg/kg	30	35				

Table 4: Effect of HAREWS on Locomotor, repetitive/ stereotypic activity

Sl .no	Treatment	Locomotory activity Scores	No. of repetitive/stereotypic activity	Duration of repetitive/stereotypic activity
1	Normal saline	295.00±4.55	0.00	0.00
2	Negative control(PPA)	167.00±3.87a***	168.00±6.08[a]***	80.33±2.30[a]***
3	HAREWS200mg/kg	234.00±6.10[b]***	48.83±2.81[b]***	18.50±0.67[b]***
4	HAREWS400mg/kg	264.30±7.77 [ns]	33.17±1.62[b]***	9.16±0.83[b]***

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph 2: Effect of HAREWS on Locomotor, repetitive / stereotypic activity

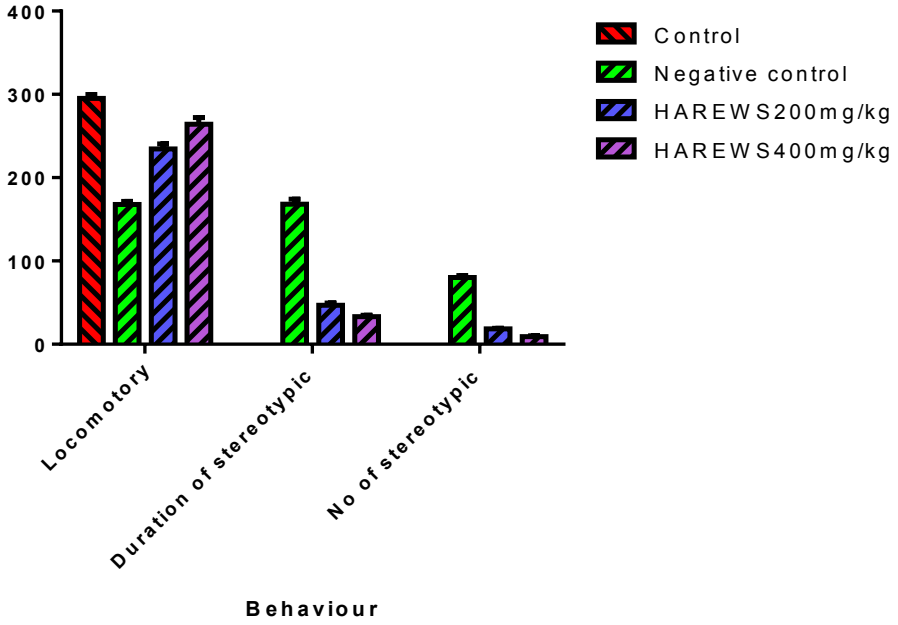


Table 5: Effect of HAREWS on Nociception thresholds

Sl.no	Treatment	Nociception thresholds
1	Normal saline	4.50±0.42
2	Negative control(PPA)	6.33±0.84 ns
3	HAREWS200mg/kg	2.83±0.30^{b}***
4	HAREWS 400mg/kg	2.50± 0.42^{b}***

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph3: Effect of HAREWS on Nociception thresholds

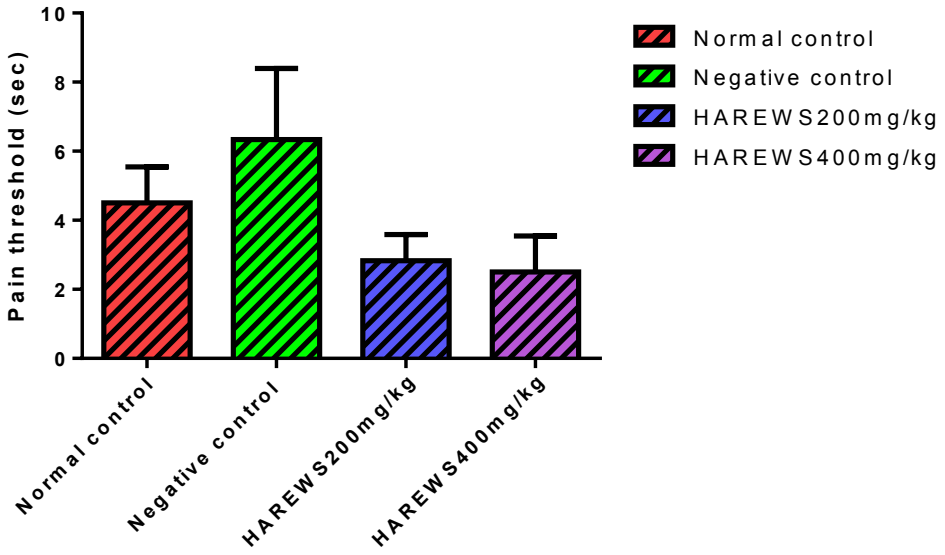


Table 6: Effect of HAREWS on object-directed behavior: percentage approach

Sl.no	Treatment	% approach towards object		
		A	B	C
1	Normal saline	44.00±2.22	26.50±2.79	29.67±2.04
2	Negative control (PPA)	54.17±1.17^{a}**	15.17±2.00^{a}**	13.17±1.01^{a}***
3	HAREWS 200mg/kg	34.33±1.35^{b}****	21.33±1.33ns	20.50±1.33^{b}***
4	HAREWS 400mg/kg	43.00±1.59^{b}***	26.83±1.35^{b}**	24.33±1.20^{b}****

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph 4: Effect of HAREWS on object –directed behavior

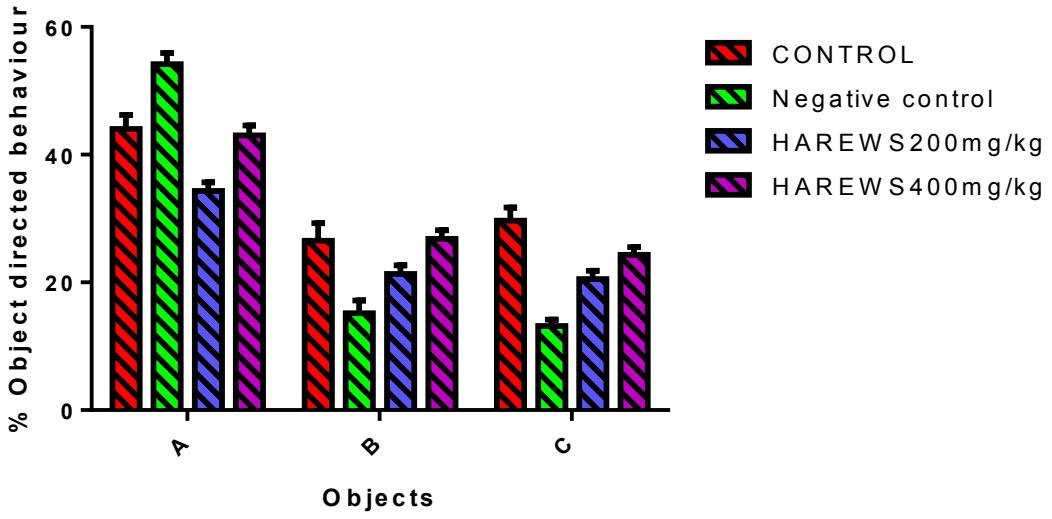


Table 7: Effect of HAREWS on Object-directed behavior: Number of sniffing bouts

Sl. No	Treatment	Number of sniffing bouts		
		A	B	C
1	Normal saline	10.33±0.67	8.33±0.55	8.50±0.42
2	Negative control (PPA)	5.55±0.67[a]***	3.66±0.21[a]***	4.33±0.30[a]***
3	HAREWS200mg/kg	7.50±0.56[b]***	5.66±0.33[b]***	5.16±0.60ns
4	HAREWS 400mg/kg	9.56±0.47[b]***	6.83±0.47[b]***	7.50±0.42[b]***

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph5: Effect of HAREWS on No. sniffing bouts

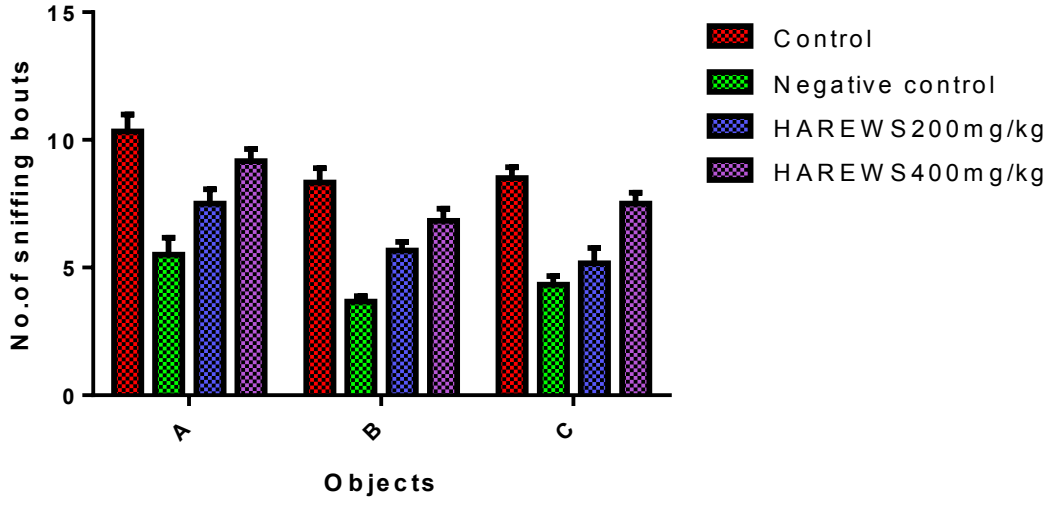


Table 8: Effect of HAREWS on object directed behavior: Time spent on objects

Sl.No	Treatment	Time spent on objects (sec)		
		A	B	C
1	Normal saline	144.20±5.97	65.57 ±1.52	59.67 ±1.54
2	Negative control (PPA)	105.70±2.71^{a}***	100.70±1.52^{a}***	73.83 ±1.04^{a}***
3	HAREWS 200mg/kg	125.00 ±1.50^{ns}	90.50±1.33^{b}***	79.50 ±0.99^{ns}
4	HAREWS 400mg/kg	105.00 ±2.63^{b}**	80.50±1.43^{b}***	87.83±2.25^{b}***

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph 6: Effect of HAREWS on object –directed behavior: Time spent on objects

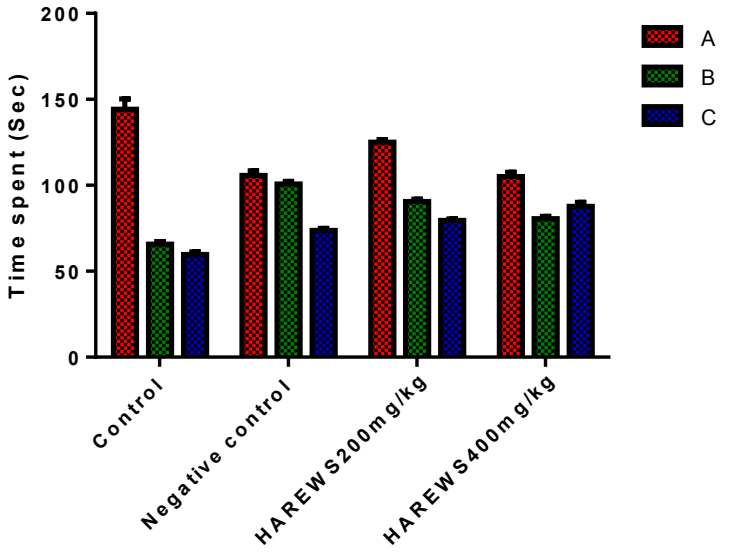


Table 9: Effect of HAREWS on Social behavior

Sl.No.	Treatment	% approach	
		Novel Mice	Novel Object
1	Normal saline	55.33±3.50	33.50±2.43
2	Negative control(PPA)	31.77±2.05[a]***	58.17±2.07[a]***
3	HAREWS200mg/kg	58.83±2.41[b]***	30.50±1.62[b]***
4	HAREWS400mg/kg	63.17±2.18[b]***	27.50±1.83[b]***

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph 7: effect of HAREWS on Social behavior

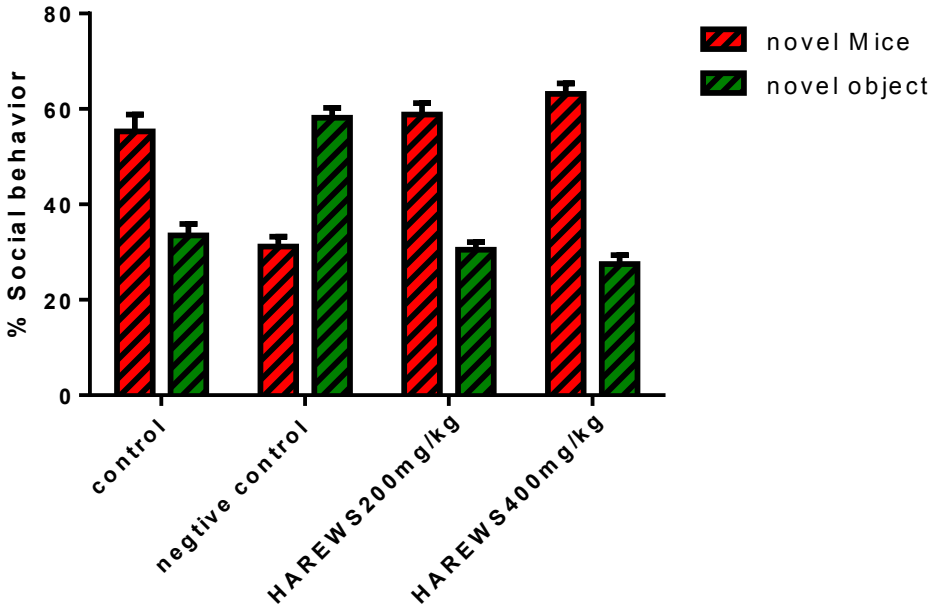


Table10: Effect of HAREWS on Elevated Plus Maze

Sl no	Treatment	No of entries	Transfer latency	Time spent
1	Normal saline	4.00±0.57	36.83±10.73	88.33±15.15
2	Negative control (PPA)	4.00±1.00^{ns}	45.00±11.71^{ns}	43.33±12.51^{ns}
3	HAREWS200mg/kg	4.50±1.05^{ns}	34.00±8.98^{ns}	81.55±12.17^{ns}
4	HAREWS400mg/kg	4.50±1.02^{ns}	58.00±15.43^{ns}	83.17±11.63^{ns}

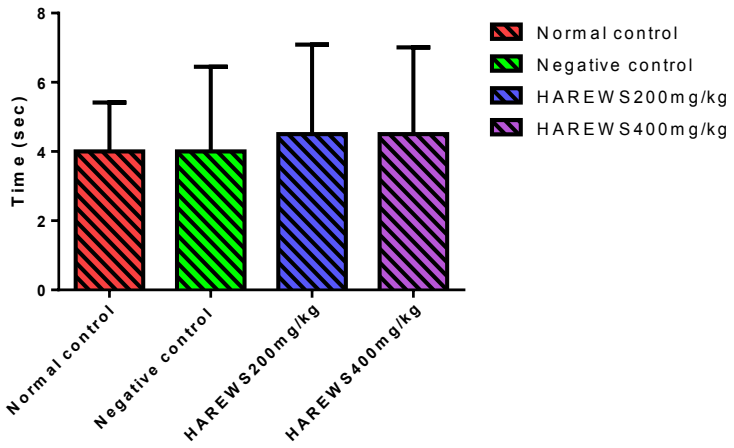
Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph 8: Effect of HAREWS on No of Entries in open arms



Graph 9: Effect of HAREWS on Transfer latency & time spent

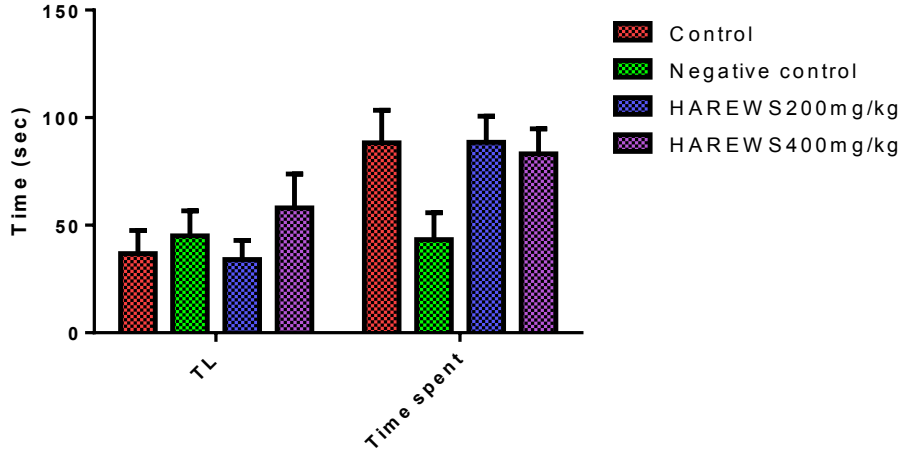


Table 11: Effect of HAREWS on Morris water test

Sl.no	Treatment	Escape Latency(sec)
1	Normal saline	15.8±2.3
2	Negative control(PPA)	59.3±7.3[a]***
3	HAREWS 200mg/kg	16.0±2.6[b]***
4	HAREWS 400mg/kg	13.0±1.6[b] ***

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph10: Effect of HAREWS on Morris water test

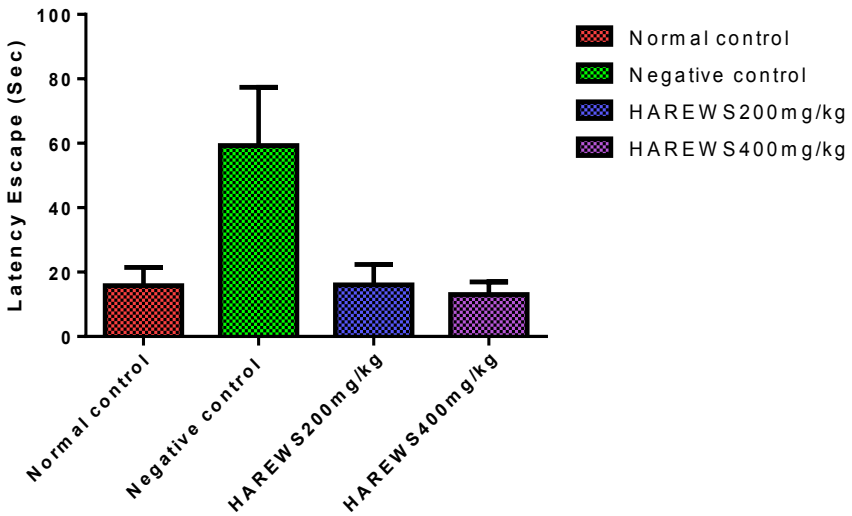


Table12: Effect of HAREWS on Serotonin

Sl.no	Treatment	Serotonin (ng/mg tissue)
1	Normal saline	12.67± 2.17
2	Negative control (PPA)	49.33±8.65[a]***
3	HAREWS 200mg/kg	19.83±2.73[b]**
4	HAREWS 400mg/kg	13.50±1.72[b]**

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph11: Effect of HAREWS on Serotonin

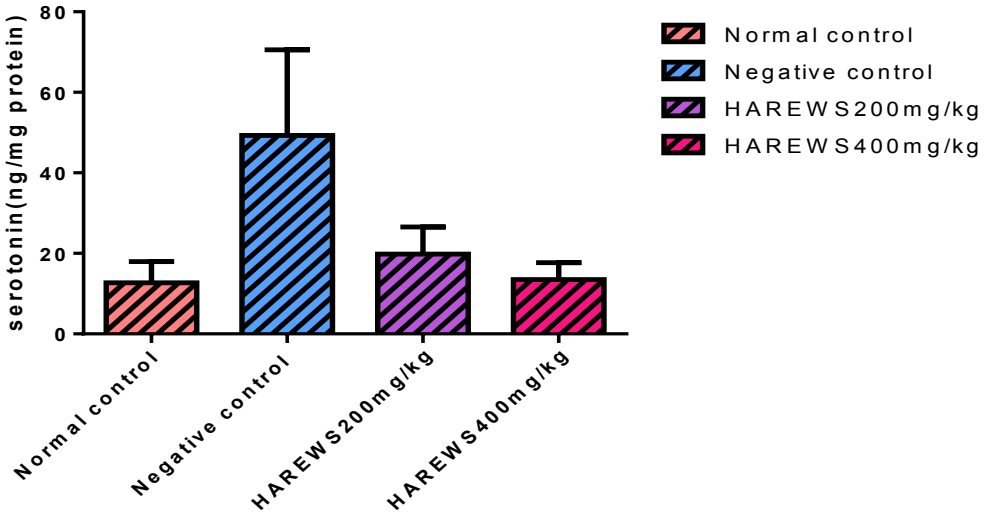


Table13: Effect of HAREWS on TNF-α

Sl.no	Treatment	TNF-α (pg/ml)
1	Normal saline	2.5 ± 0.77
2	Negative control (PPA)	3.15±0.08[a]***
3	HAREWS 200mg/kg	1.41±0.011[b]***
4	HAREWS 400mg/kg	1.77±0.031[b]***

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph12: Effect of HAREWS on TNF-α

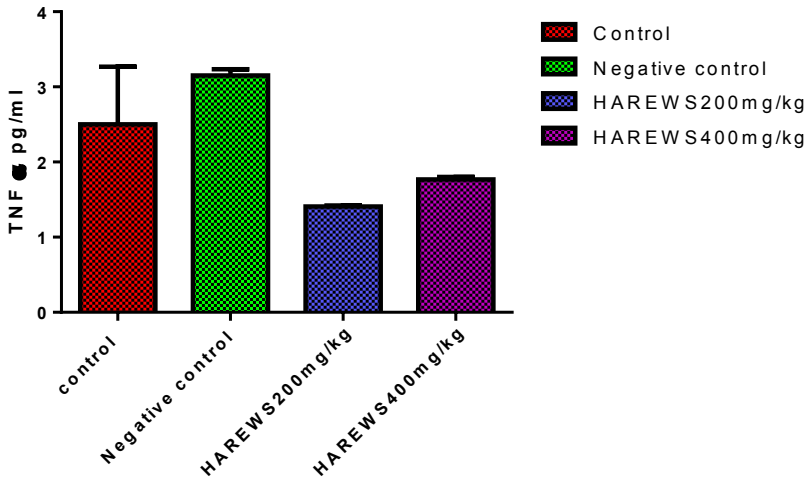


Table14: Effect of HAREWS on Interleukin-6

SLNo.	Treatment	Interleukin
1	Normal saline	2.16±0.45
2	Negative control (PPA)	2.54±0.31^{ns}
3	HAREWS200mg/kg	1.61±0.39**
4	HAREWS400mg/kg	1.46±0.26^{ns}

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph13: Effect of HAREWS on IL-6

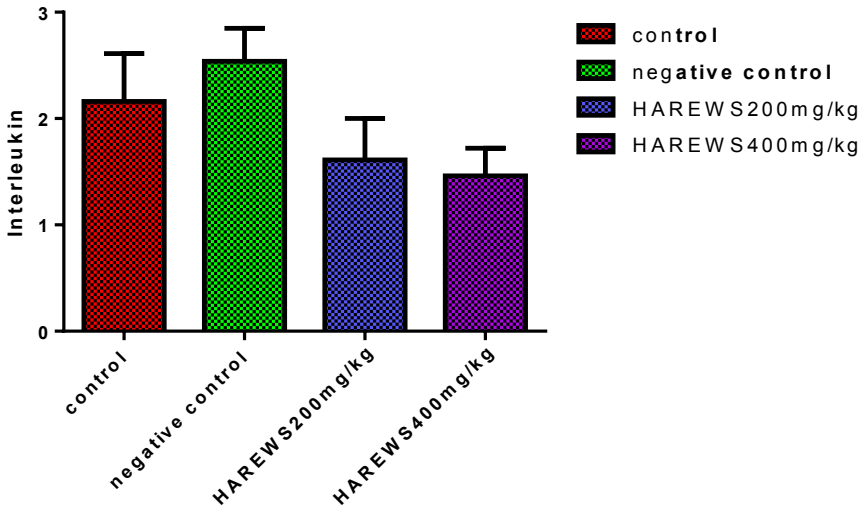


Table15: Effect of HAREWS on Superoxide dismutase

Sl.no	Treatment	SOD (Units mg/wet tissue)
1	Normal saline	5.96±0.34
2	Negative control(PPA)	3.54±0.19 a***
3	HAREWS200mg/kg	4.52±0.20 ns
4	HAREWS 400mg/kg	5.22±0.09ns

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph14: Effect of HAREWS on Superoxide dismutase

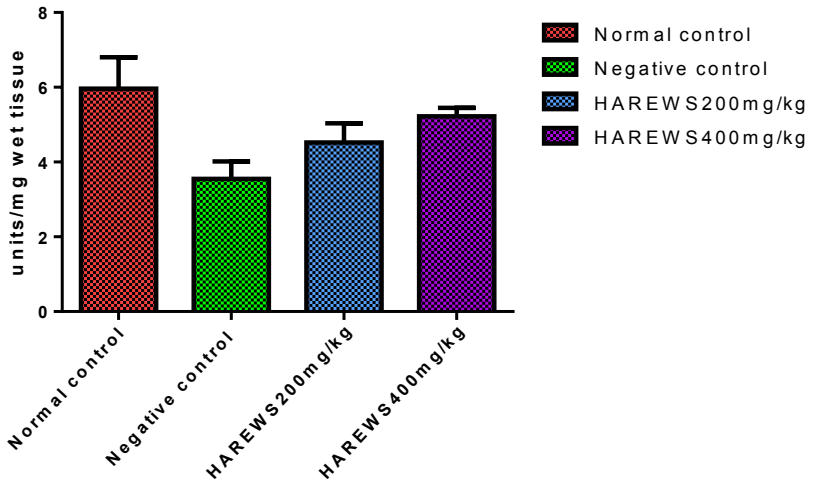


Table16: Effect of HAREWS on Catalase

Sl.no	Treatment	Catalase (Units mg/wet tissue)
1	Normal saline	6.25±0.17
2	Negative control(PPA)	3.47±0.15[a] ***
3	HAREWS 200mg/kg	4.79±0.27[b]***
4	HAREWS 400mg/kg	5.83±0.22[b]***

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph15: Effect of HAREWS on Catalase

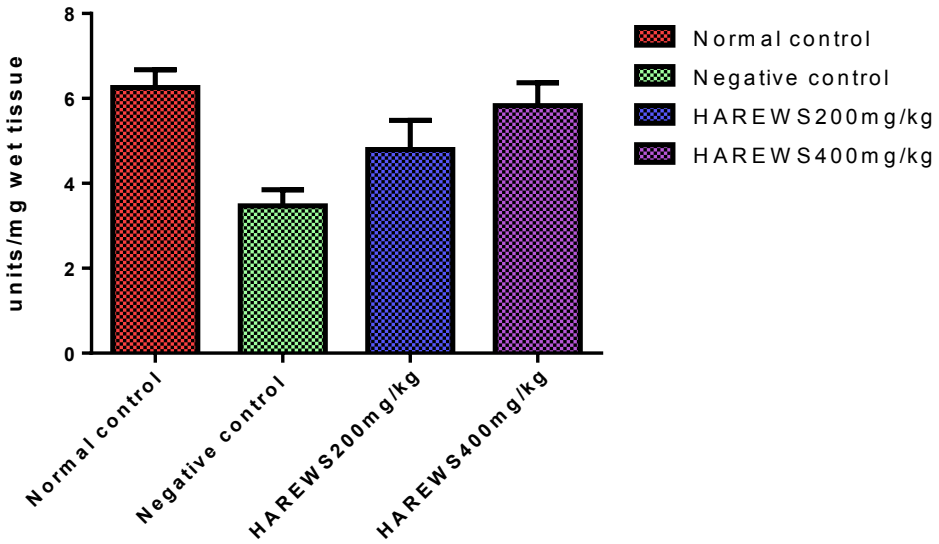


Table17: Effect of HAREWS on Lipid peroxide

Sl.no	Treatment	Lipid peroxide (Units mg/wet tissue)
1	Normal saline	6.84±0.38
2	Negative control(PPA)	3.88±0.19[a]***
3	HAREWS 200mg/kg	6.16±0.18[ns]
4	HAREWS 400mg/kg	5.96±0.37[ns]

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph16: Effect of HAREWS on Lipid Peroxide

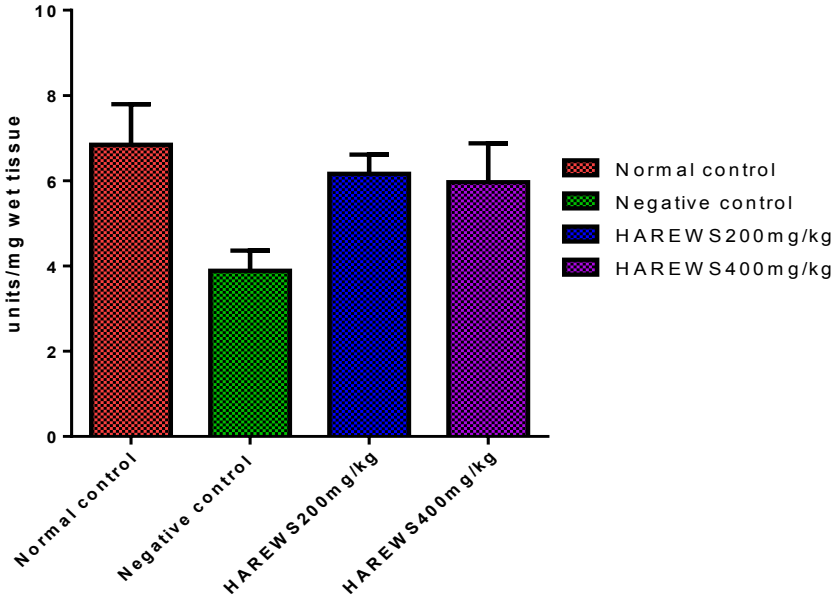


Table 18: Effect of HAREWS on Glutathione Peroxidase

Sl.no	Treatment	Glutathione peroxidase (Units mg/wet tissue)
1	Normal saline	6.85±0.20
2	Negative control(PPA)	3.07±0.28[a]***
3	HAREWS 200mg/kg	5.56±0.20[b]***
4	HAREWS 400mg/kg	6.56±0.14[b]***

Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph 17: Effect of HAREWS on Glutathione peroxidase

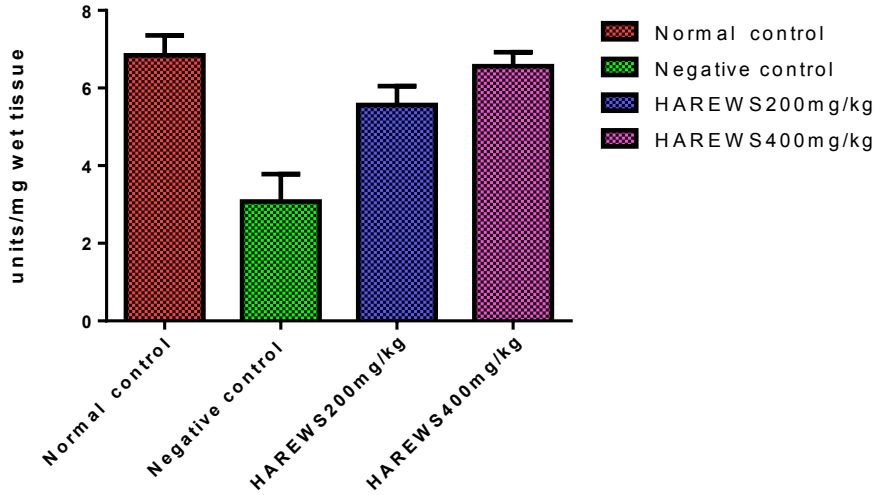


Table 19: Effect of HAREWS on Glutathione Reductase

Sl.no	Treatment	Glutathione reductase (Units mg/wet tissue)
1	Normal saline	6.75±0.19
2	Negative control(PPA)	3.25±0.15[a]***
3	HAREWS 200mg/kg	4.80±0.153[b]***
4	HAREWS 400mg/kg	5.72±0.22[b]***

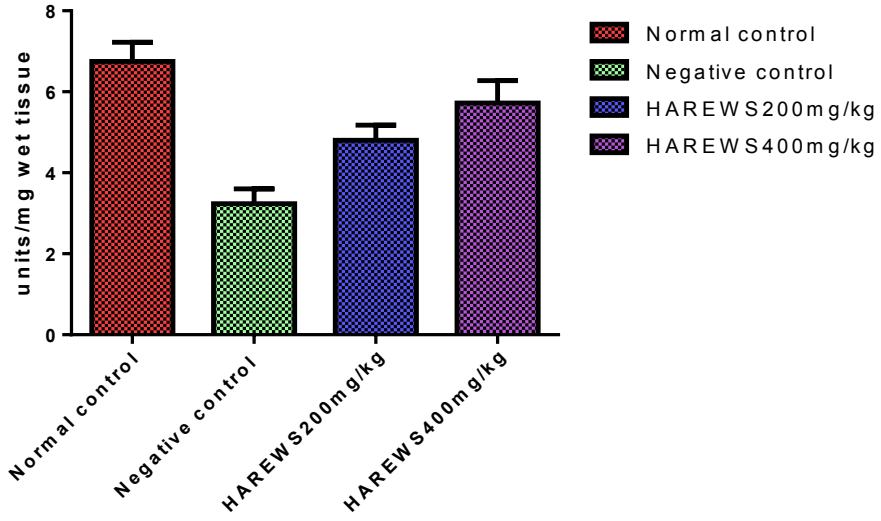
Values are represented in Mean ± SEM, n=6

Comparison: a- Group I vs Group II, b- Group II vs Group III and Group IV

Statistical significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

ns- Non significant, *p<0.05, **p<0.01, *p<0.001

Graph18: Effect of HAREWS on Glutathione Reductase



7.10 HISTOPATHOLOGY

Fig 8a: control group

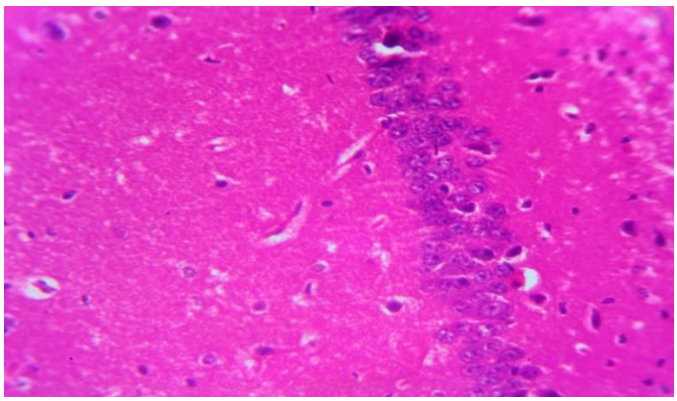


Fig8b: Negative control

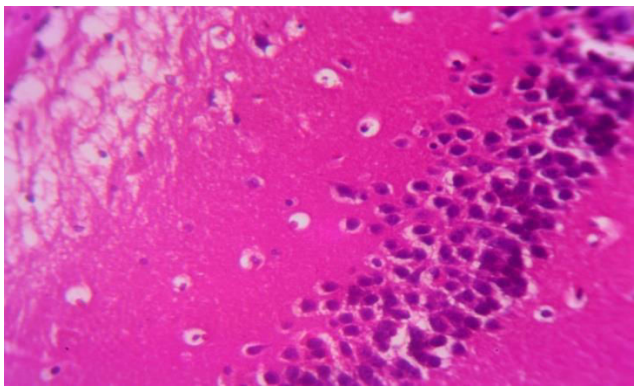


Fig8c: HAREWS200mg/kg

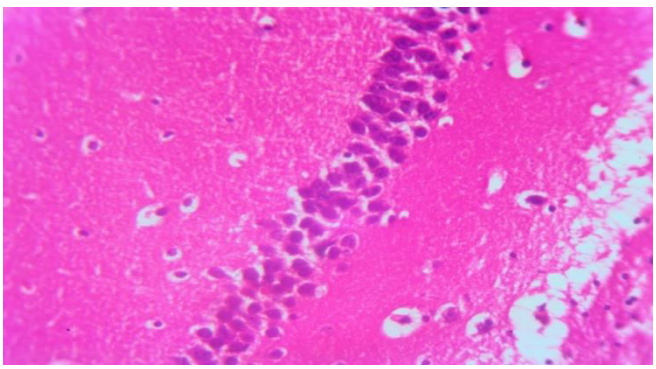
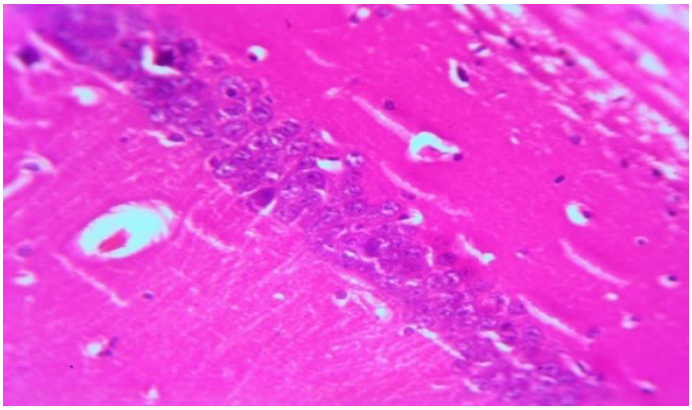


Fig8d: HAREWS400mg/kg



8. DISCUSSION

Autism is a neurodevelopment disorder which is characterized by impaired social interaction, verbal and non-verbal communication and repetitive behavior. The investigative criteria require that symptoms became apparent in early childhood, typically before three year age. Autism is highly heritable diseases, researcher suspect both genetic fact as well as environmental fact as causes, in rare case autism is strongly related with agent that causes birth defect.

ASD is also known as pervasive developmental disorder which includes Asperger's syndrome, Rett's syndrome, Childhood Disintegrative Disorder, Autistic disorder and PDD-NOS (Pervasive Developmental Disorder Not Otherwise Specified). It is characterized by impairments in three domains, specifically communication, social interaction and behaviors that are restricted and repetitive in nature.

Childhood is a key developmental phase; with exacerbation of many ASD linked symptoms. Propionic acid is connected with ASD. A separation of ASD patients with gastrointestinal symptoms and behavioural regression have superior levels of Clostridia, an early gut colonizer known to produce PPA and other short chain fatty acids. ICV injections of PPA on rodent's brain induced repetitive behaviours, hyperactivity, turning behaviour, retropulsion, caudate spiking, kindled seizures, impaired social behaviour, poor memory, increased oxidative stress markers, and induced an inherent neuroinflammatory response all of which appear consistent with findings from ASD patients.

The present study has exposed the therapeutical effect of HAREWS on object directed behaviour, social behaviour and neuro-inflammation against PPA induced ASD in mice PPA induced ASD was assessed by various parameters such as locomotory activity, nociception threshold, object directed behaviour, novel mice vs. novel object directed behaviour, Elevated plus maze, Morris Water maze test.

Test on open field measured the locomotory activity, repetitive/stereotypic behaviour of mice. The animals that are induced with ASD by propionic acid showed staggering and toe walk. The animals are found to hyperactive with repetitive/stereotypic activity. HAREWS treated animals showed the reversed behaviours.

Nociception threshold is analysed by tail flick method where the tail of animals were exposed to the high temperature water to induce pain. Animals which are normal respond more quickly to the pain. Mice with ASD showed delayed response to the heat as seen in ASD patients who possess high threshold to pain. Animals treated with HAREWS showed usual threshold like control mice representing that drug maintains the normal response to pain sensitivity

Social behaviour of animals was tested by exploring the same with novel mice and an object. Normal mice are always expected to explore the novel mice rather than the object. Mice of PPA treated groups does not approached novel mice as they lack social interaction. Those that are pretreated with HAREWS showed significant increase in their interaction with novel mice as like normal mice, indicating that the drug under the present study improves the social interaction.

Elevated plus maze task has been extended to measure the anxiety behaviour of the animal. The method was based on the fact that sudden exposure of animals to the higher altitude in open field induces anxiety, where the normal animals preferentially seek the closed arms than the open arm. Animals that are induced with ASD did not showed significant increased or decreased anxiety when compared to normal mice.

The Morris water maze represents a more specific test of spatial memory. The essential feature of this technique is that mice were placed into a large circular pool of water and can escape onto a hidden platform. Since the platform offers no spatial cues to guide escape behaviour and the mouse can escape only from swimming by climbing onto the platform apparently learns the spatial location of the platform where ever the starting position may be. The only spatial cues are those outside water tank are primary visual cues. Thus, the flexibility of the task makes it a widely adequate experimental model for the assessment of cognitive skills. Typically, ASD mice took much time for escape indicating the lack of remembering visual cues to escape onto the platform. Such a diminished cognition was reversed by the administration of HAREWS and exhibited decreased escape latency (EL), indicating the well developed spatial memory inspite of PPA induced cognition deficits.

Serotonin is the critical neurotransmitter modulating short term habituation. Decreased level of serotonin is associated with repetitive/stereotypic behaviour and feeling of unhappiness. Serotonergic effects have also been detected on the region implicated in memory storage. Brain of ASD mice was observed to have decreased level of serotonin. Those that are treated with HAREWS increased brain serotonin level indicating the improvement in repetitive behaviour and cognition skill.

TNF-α is a pro-inflammatory cell signaling protein involved in inflammation and is one of the cytokines that make up the acute phase reaction. It is produced chiefly by activated macrophages, although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells, neutrophils, mast cells and neurons. Mice with ASD showed significant increase in brain TNF-α level indicating neuro-inflammation. Group of animals that are pretreated with HAREWS reduced the TNF-α level that indicates that it possesses anti-inflammatory activity.

Interleukin-6 is a pro-inflammatory cytokines has an important role in immunity. IL-6 induces growth and terminal differentiation of β-cells; secretion of immune-globulins, differentiation and activation of T-cells & macrophage. Many types of cells including T-cells, fibroblast & endothelial cell produce IL-6 to stimuli such as bacteria, viruses and other cytokines. Mice with ASD showed significant increase in brain IL-6 level indicating neuro-inflammation. Animals which are pretreated with HAREWS reduced the IL-6 level that indicates it possess the anti-inflammatory activity.

The brain is highly sensitive to oxidative damage because of its high oxygen consumption. Thus, oxidative biomarkers such as superoxide dismutase (CAT), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRD), lipid peroxidase (LPO) are analysed to study the severity of the disorder and effect of the drug on it. Level of SOD, CAT, GPx and GRD were found to be decreased ASD affected mice and LPO was observed to be high in its concentration when compared to normal mice. The mice those were treated with HAREWS were observed to possess antioxidant activity showing the level of above mentioned oxidative biomarkers similar to normal mice.

9. SUMMARY

The preliminary phytochemical studies on HAREWS discovered the presence of various phytoconstituents such as alkaloid, carbohydrates, glycosides, steroid, proteins, flavonoids and saponin.

The habituation behavior of HAREWS on PPA induced ASD assessed by objective directed behavior; novel mice vs. novel object directed behavior, nociception threshold, elevated plus maze task and cognition development assessed by Morris water maze task.

The habituation behavior on open field revealed that HAREWS significantly reversed stereotypic and staggering movement. Memory retention was assessed on Morris water maze and HAREWS found greatly improved/enhanced the spatial memory. On HAREWS treatment the pain threshold was maintained as like normal subjects. Both propionic acid and HAREWS has found not to possess significant anxiety activity.

The biochemical changes that are responsible for stereotypic activity, cognition impairment, and neuro inflammation were assessed by estimating serotonin, TNF-α, Interleukin-6 respectively. HAREWS provided favorable effect on these above biochemicals.

The antioxidants defense in the brain tissues were estimated in terms of SOD, CAT, LPO, GPx, GRD and HAREWS increased the antioxidant levels at respective dose leading to reduce oxidative stress. This clearly indicates the potential of the extract to delay the generation of free radicals that cause neuronal damage.

The histopathology of ASD mice showed increased in neuron number and reduced size as seen in ASD patients. Whereas the control and HAREWS treated mice showed normal level of neuronal size and counts.

10. CONCLUSION

The present study demonstrated that ICV-infusion of propionic acid induced autism spectrum disorder in mice. Hydro-alcoholic root extract of *Withania somnifera* helps in improving object directed behavior, social behavior and cognitive skill. And also improve the neuro-inflammation. It also showed the anti-oxidant property which act as a neruo-protective.

Further studies are expected on the isolated components of the extract in order to understand the exact mechanism of its action and also studies at cellular level changes by more sophisticated methods for investigation.

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